UNIVERSITY OF LIVERPOOL

IS THE CIRCADIAN CLOCK AN IMPORTANT ADAPTIVE TRAIT IN BARLEY PLANTS?

By

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A thesis submitted in fulfilment of the degree of Doctor of Philosophy

School of Biological Sciences

June 2012

DECLARATION

I, Radia Salem, confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

Signed **Dated: June 2012**

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ABSTRACT

Circadian rhythms are biological oscillations that continue to run under constant conditions with a periodicity close to 24 h. The clock allows the organism to anticipate the regular changes in the light and temperature in daily and seasonal environmental cycles that occur as a result of the earth's rotation on its axis. In the higher plants, there are a wide range of biological processes that are controlled by the circadian clock, such as leaf movement, cell growth, flowering time, metabolism, respiration, photosynthesis and approximately 30% of gene expression in *Arabidopsis thaliana*. It has been revealed that accurate and robust endogenous circadian clocks that can be synchronised to external time cues provide an adaptive advantage and increase the vegetative growth in *Arabidopsis* plants.

The plant circadian clock has been thoroughly researched using the model higher plant *A. thaliana*. Isolating and modelling several clock components has revealed a series of interlocking feedback loops at the heart of the molecular mechanisms of the clock. It has been demonstrated through phylogenetic analysis that there is a higher degree of conservation of the clock genes within Angiosperms. In this research, existing knowledge from studying the *Arabidopsis* circadian clock was used to identify and further understand the importance of the clock in the barley plants.

Different strategies were implemented to investigate the function of barley oscillator genes including silencing evening genes (*TIMING OF CAB EXPRESSION 1(TOC1)* and *GIGANTEA (GI)* using RNA-interference (RNAi) techniques. Results point towards the conclusion that losing the full functionality of *HvTOC1* and *HvGI* may have a major effect on plant growth and development. Research demonstrated that *HvGI* over-expressed in *A. thaliana* plants completely rescued the late flowering phenotype of the gi-11 mutant under long day conditions, thus confirming that the *HvGI* gene is an *Arabidopsis GI* gene homologue, functioning as a regulator in both the circadian clock oscillation and the photoperiodic pathway.

A delayed fluorescence (DF) protocol allowing the measurement of robustness and accuracy of the barley circadian clock was developed and optimised. This protocol was

also used to investigate whether the *PHOTOPERIOD-H1* (*PPd-H1)* gene is the barley *PSEUDORESPONSE REGULATORS 7 (PPR7)* gene by comparing the DF oscillation pattern of *PPd-H1* wild type and a *Ppd-H1* mutant with transgenic lines of over-expression *PPd-H1* in Golden Promise barley. Indeed, the results confirm that *HvPPDH1* is not analogous to *AtPRR7* but perhaps to *Arabidopsis PSEUDORESPONSE REGULATORS 3* (*AtPRR3).* This study is an initial step to identifying and understanding how the clock works in a crop species and whether the clock is an important agronomical trait.

ACKNOWLEDGMENTS

The journey undertaken when conducting a PhD is a long and arduous one and my own work has made me come across difficult times. However, the support I have received has made me able to achieve the final submission of this thesis.

Firstly, I should start in the name of God since everything we do or come across is related to his will. Therefore, I have to admit that it is only through the light of God Almighty that I have been able to achieve this stage, which, on many occasions, I have felt would never come. Fortunately, I have been provided with the support, motivation, knowledge, friendship and love to make the trip through the long research and writing process more bearable and rewarding.

I am grateful to my supervisor Dr Anthony Hall for offering me this PhD opportunity on circadian biology: his excellent supervision, understanding, faith, patient, advice and constant guidance from the very early stage of this research, as well as giving me most extraordinary experiences throughout the project lab work. Above all, and the most needed, was his genuine caring and concern, encouragement and support in various ways, and without his exceptional inspiration, this thesis would not have been completed. I wish also to express my sincere thanks to my second supervisor James Hartwell for supervising this PhD project.

I would like to express my sincere appreciation and gratitude to Dr Pete Gould and Dr Sean Cotter for instructing me on how circadian tools work, answering countless questions and for their scientific advice. Special thanks go to Jean Woods for all her help and support, she made my work so much enjoyable. I am indebted to her help more than she knows. Furthermore, many thanks and blessings to all people working in lab G, especially,

Nicola and Hayeit, the most dedicated and generous people that I have ever met and I feel fortunate and honoured to have been working with them in such a friendly environment. Thanks also to staff and members of the School of Biological Sciences, especially the secretary Annie, Dr Meriel Jones, and Prof Martin Mortimer for annual review of this project's progress. It has been an unforgettable experience and great privilege to spend several years in the University of Liverpool.

I am delighted to thank both Dr Wendy Harwood and Dr Mark Smedley from the John Innes Centre for their teaching and for helping me with the barley transformation technique. I would also like to thank Prof Joanna Putterill from the University of Auckland for providing me with *GI-11-ox* (35S:*GI*) seeds. I am also deeply grateful to Dr David Laurie from the John Innes Centre for providing me with ppd-h1-ox lines seeds.

I am deeply and forever indebted to my parents, for their enormous love, prayers, support and encouragement throughout my entire life. I am very grateful to my brothers and sisters for their inspiration and support which forged my desire to achieve my life goals. Many thanks to my friends for their support as well as to those who have profoundly influenced me and helped me in getting to this point. Finally, special thanks are extended to staff and members of the University of Tripoli and the Ministry of Higher Education, Libya for providing the financial support for this PhD training in research.

ABBREVIATIONS

CaMV	Cauliflower mosaic virus
CCA1	*CIRCADIAN CLOCK ASSOCIATED 1*
ccdB gene	Control of Cell Death B gene
DD	Constant Dark
DF	Delayed fluorescence
dsRNA	Double-stranded RNA
EE	Evening element
FLC	*FLOWERING LOCUS C*
FRI	*FRIGIDA*
GI	*GIGANTEA*
GP	Golden Promise line
Hyg	Hygromycin
HvCCA1	Barley CCA1 gene
HvGI	Barley GI gene
HvTOC1	Barley TOC1 gene
LAR	Leaf area ratio
LD	Long days
LHY	*LATE ELONGATED HYPOCOTYL*
LL	Constant light
LSD	Least significant difference
LUC	Firefly *LUCIFERASE*
LWR	Leaf weight ratio
MS	Murashige and Skoog

OX	Over-expression
PCR	Polymerase chain reaction
PDWT	Ppd-H1- wild type line
PDX	ppd-H1 mutant
phyB	Phytochrome B signalling
PPDH1	*PHOTOPERIOD-H1*
PRR	Pseudo response regulator
PRR3	*PSEUDORESPONSE REGULATORS 3*
PRR7	*PSEUDORESPONSE REGULATORS 7*
PSII	Photosystem II
RAE	Relative amplitude error
RB	Red blue light
RGR	Mean relative growth rate
SD	Short days
SE	Standard error
SLA	Specific leaf area
T cycle	Light-Dark cycle
T	Transformant
TOC	*TIMING OF CAB*
Ubi-1	Maize ubiquitin gene
ULR	Unit leaf rate
WT	Wild type
ZTL	*ZEITLUPE*

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CHAPTER 1: LITERATURE REVIEW OF THE PLANT CIRCADIAN CLOCK

1.1 Circadian rhythms

Circadian rhythms are biological oscillations that continue to run under constant environmental conditions with a periodicity close to 24 h. These rhythms are found in most animal and plant species. The clock allows organisms to predict the regular changes in the environment that happen as a result of the earth's rotation on its axis (Ding *et al.*, 2007). Circadian rhythms can be entrained by environmental cues such as light and temperature and their phase can be shifted by exposure to a pulse of input stimuli, consequently resetting the clock. Moreover, the period of the circadian rhythms is temperature compensated; this allows a circadian clock to maintain robust and accurate timing over a broad range of physiological temperatures (Bunning, 1971; McClung, 2006). In higher plants, a large amount of biological processes are controlled by the circadian clock, such as the movements of leaves and petals, cell growth and calcium level, stomata opening, stem elongation, respiration and photosynthesis, and expression of a huge number of different genes (Dodd *et al.*, 2005; Murakami *et al.*, 2007). It has also been noted that expression of genes regulated by the circadian output pathway peaks at different times of the day. Many of these genes are related to photosynthesis, such as the *CHLOROPHYLL A_B BINDING PROTEIN* (*cab)* and *CATALASE2 (cat2)* genes that expressed early in the subjective day, while *CATALASE3 (cat3)* expression peaked late in the afternoon (Johnsson and Engelmann, 2008)

Conceptually, the general circadian system can be separated into three main parts: the input pathways, the central oscillator and the output pathways (Figure 1.1A) (Barak *et*

al., 2000; McClung, 2000). The input pathways adjust the phase of the rhythm to environmental cues such as light and temperature, and allow for perception of both daily and seasonal information. The central oscillator generates and maintains rhythmicity, interprets environmental information and transmits signals to a diverse set of multiple outputs pathways. The core of this central oscillator is typically composed of multiple interlocked positive/negative feedback loops that involve transcriptional activation and repression (Millar, 2004). Oscillating patterns are created by transcription factors that activate the expression of clock genes that negatively regulate their own transcription. Lastly, there are output pathways that generate physiological events (Barak *et al.* 2000; Harmon *et al.* 2005; Ruoff *et al.*, 2005; Yakir *et al.*, 2007). Such a depiction is of the traditional three-component model of the circadian system. It is an over-simplification because the clock influences the ability of light and temperature to reset the clock phase. Moreover, the clock input signalling pathways, such as some photoreceptors, may also be regulated directly by clock outputs, and the clock components might be operated both within the central oscillator and in input and output pathways, thus this results in a more complicated model (Figure 1.1B) (Harmer, 2009). Despite the increasingly intricate picture of the circadian clock model, this model is useful as a fundamental working system that can be used to explain the concept of the clock. Work over the last decade now aims to produce a mathematical model of the clock, allowing us to better understand this complex system and make predictions regarding its structure and function (Pokhilko *et al.*, 2012).

A long-standing question arises as to whether there is one or multiple circadian clocks operating within a single organism and possibly even within the same cell and how these oscillators are coordinated with each other in one circadian system. It seems

that the circadian rhythms are cell autonomous; consequently, multicellular organisms indeed include multiple clocks. In animals, for example, circadian systems operate with a single master clock found in the brain that controls the activity rhythms for the entire organism (Nakamura, 2010). In comparison, Thain *et al.* (2000) reported that plants are believed to possess a circadian oscillator in every cell that can respond independently to environmental signals perceived by that particular cell. Comparison of clock regulation of the *CHLOROPHYLL A_B BINDING PROTEIN 2* (*CAB2*) and *CATALASE3* (*CAT3*) promoters also suggests they are perhaps driven by two distinct oscillators within the single cell with different responses to temperature signals (Michael *et al.*, 2003). Hall *et al.* (2002) indicated that the free running periods of *phyB* and *CAB2* rhythms in *Arabidopsis* seedlings are generated by different clocks, though also possibly driven by different clocks in different cell types. Moreover, measuring rhythmicity in individual leaf cells in intact living *Arabidopsis* plants using fluorescence-tagged *CCA1* has shown that stomatal guard cells may have a longer period and lower amplitude of circadian rhythms than those in surrounding mesophyll and epidermal cells. In constant light conditions, *CCA1* mRNA levels cycle with a longer period and lower amplitude in guard cells than in whole leaves, while GI transcript levels appear mostly undisturbed. Levels of *TOC1*, *CHE* and *LHY* mRNA were greater damped in guard cells compared with whole leaves (Yakir *et al.*, 2011). Recent studies suggest that there is a possible specialisation of circadian clock function in specific tissues, including in root and vascular tissue systems (James *et al.*, 2008; Para *et al.*, 2007). In continuous light, *TOC1 mRNA* clearly cycles in the shoots but not in the roots. *LHY* and *CCA1* are normally regulated by *TOC1* oscillations by binding directly into the evening element (EE) in its promoter. However, in roots *LHY* is incapable of binding the EE, resulting in constant and raised *TOC1 mRNA* levels consistent with the lack of function of the evening loop in the roots. Furthermore,

the period of *LHY* expression was shortened in the shoots of the *TOC1* mutants but not in the roots (James *et al.*, 2008). The expression of the *PRR3* gene was found to mainly accumulate in the vascular tissue of the leaves. it was also found that the mutation in this gene seems to have a strong effect on clock-regulated genes that are particularly expressed in the vascular tissues (Para *et al.*, 2007).

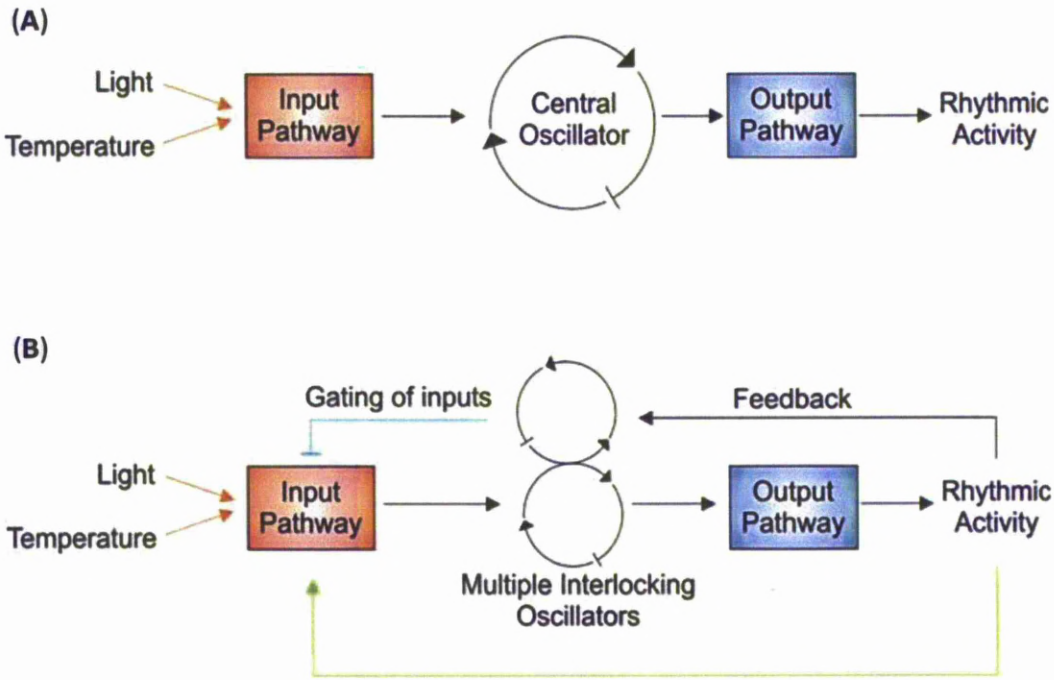


Figure 1.1. Illustration of the circadian clock system. (**A**) Model of a simple circadian system split into three discrete components: input pathway, a central oscillator and an output pathway. (**B**) An elaborated description of the clock complex network, consisting of the multiple functions of the core clock genes, performing both within the oscillator and in clock input and output signalling pathways. Input pathways can be regulated by clock outputs' components. Likewise, input pathways can adjust multiple clock genes and directly affect clock outputs (Gardner *et al.*, 2006).

1.2 The oscillator of the *Arabidopsis* circadian clock

In the past decade, identification of the molecular components of the plant circadian oscillator has been performed using the model higher plant *Arabidopsis thaliana.* Several putative circadian clock-associated genes have been recognised through intensive mutational analysis and clock controlled promoters. The best candidates for the central oscillator components are morning-expressed *LATE ELONGATED HYPOCOTYL (LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* genes. These homologous transcription factors include a single MYB binding domain DNA. These transcription factors bind to the evening element and regulate the evening-expressed *TIMING OF CAB EXPRESSION 1 (TOC1)* gene. *TOC1* belongs to the Pseudo-response regulators' family, also known as a *PSEUDO-RESPONSE REGULATOR 1 (PRR1).* Together, they regulate each other's expression inversely to form a main negative feedback loop (Alabadi *et al.*, 2001; Salome and McClung, 2004; Murakami *et al.*, 2007).The *CCA1* and *LHY* proteins bind directly to the *TOC1* promoter and inhibit its expression, while, on the other hand, the *TOC1* protein acts indirectly via an unknown component X to activate *LHY/CCA1* expression. The accumulation of *LHY/CCA1* represses *TOC1* expression, which leads to lowered activation of *LHY/CCA1.* As a result, a decrease in *LHY/CCA1* expression allows *TOC1* transcript levels to increase and reach a maximum at the end of the day (Locke *et al.,* 2005), since *TOC1* lacks a DNA-binding domain and its transcript expression peak does not match the time of the *CCA1/LHY* peaks. Therefore, a TCP transcription factor *CCA1 HIKING EXPEDITION* (*CHE*) protein could be partially responsible for the role of the transcriptional activator X that directly binds to the *CCA1* promoter to regulate its expression (Pruneda-Paz *et al.*, 2009). Over-expression of *CHE* repressed the expression of *CCA1*, and *CCA1* expression was increased in *che* mutants. The *CCA1* expression is inhibited by *CHE*, whereas *CCA1* negatively regulates expression of *CHE,* adding an

extra *CCA1/CHE* feedback loop to the original core loop (Figure1.2). *CHE* was also found to be directly interacting with *TOC1*, and both proteins are associated with the same region of the CCA1 promoter, establishing a molecular link between TOC1 protein levels and CCA1 expression (Figure1.2). However, *CHE* functions as a repressor rather than an activist and also does not bind to the *LHY* promoter, thus additional activators and repressors connecting *TOC1* to *CCA1/LHY* have not yet been identified (Pruneda-Paz *et al.*, 2009).

Previous studies found that the flowering-time gene *GIGANTEA (GI)* works alongside *TOC1* to compose a secondary feedback loop (Locke *et al.*, 2005; Mizoguchi *et al.*, 2005). The *GI* gene partially fulfils the component Y function and is able to activate *TOC1* expression, and in turn TOC1 later represses *GI.* Expressions of both TOC1 and GI genes peak in the evening and appear to function as positive regulators of *CCA1/LHY* genes (Locke *et al.*, 2005; Mizoguchi *et al.*, 2005). In contrast, Ito *et al.* (2008) reported that *GI* is not essential for the transcriptional activation of *TOC1,* and its transcription is not negatively regulated by only *TOC1* protein, but also both of them play coordinated and positive roles in regulated expression of the core clock morning genes *CCA1* and *LHY.* Thus, they suggested that *GI* is not suited to fulfil the Y role but also plays complicated clock-associated roles (Ito *et al.*, 2009). Furthermore, *PSEUDORESPONSE REGULATORS 7 and 9 (PRR7 and PRR9)* both appear to function as part of the oscillatory mechanism with *CCA1/LHY* to constitute a tertiary feedback loop resulting in a three-loop circuit (Farre *et al.*, 2005). Both *PRR7* and *PRR9* act as negative regulators to the *CCA1/LHY* genes while *CCA1/LHY* promote expression of *PRR7* and *PRR9* (Locke *et al.*, 2006). The three loops model (Figure1.2) suggests that the morning-expressed

PRR7/9–LHY/CCA1 loop and the evening-expressed *TOC1–Y/GI* loop are combined together by the central loop *LHY/CCA1–TOC1– X* (Locke *et al.*, 2006).

Another *TOC1* homolog, *PRR5*, has recently been found to be associated with the *CCA1* and *LHY* promoters in vivo and directly down regulates *CCA1* and *LHY* expression (Nakamichi *et al.*, 2011), while expression of *PRR5* itself is regulated by *REVEILE8 (RVE8)* (Rawat *et al.*, 2011). The *RVE8* is one of the *REVEILE (RVE1-8)* clock regulator genes that belong to a subfamily of MYB transcription factors such as *LHY/CCA1*. *RVE8* directly promotes expression of PRR5; in turn, *PRR5* represses expression of *RVE8*, forming a negative transcriptional feedback loop that acts within the plant's circadian network (Rawat *et al.*, 2011). All *REVEILE* proteins bind with high affinity to the evening element (EE) promoter, except *RVE5* (Andersson *et al.*, 1999).

Other clock-associated genes identified as *EARLY FLOWERING*3 and 4 *(ELF3* and *ELF4), ZEITLUPE (ZTL),PSEUDORESPONSE REGULATOR 3 (PRR3) and LUX ARRHYTHMO (LUX)* also known as *PYTOCLOCK1(PCL1)* have not been included in the three loops model despite their critical role in the *Arabidopsis* central clock (Murakami *et al.*, 2007). *ELF3* and *LUX* are also evening-expressed putative transcription factors that directly repress the expression of *PRR9* through binding to its promoter. *LUX* also binds to its own promoter and *ELF3* negatively influences expression of *PRR7, GI* and *TOC1* (Dixon *et al.*, 2011; Helfer *et al.*, 2011). It has been proposed that the evening phase gene *ELF4* might form another negative feedback loop with *CCA1/LHY. ELF4* activates expression of *CCA1/LHY* and represses *TOC1*, whereas expression of *ELF4* is negatively regulated by *CCA1* and *LHY* (Kikis *et al.*, 2005; McWatters *et al.*, 2007). *ELF4* additionally was shown to be required for red light mediated induction of *CCA1/LHY* and maintained rhythmicity in the central loop under constant light conditions

(Doyle *et al.*, 2002; Kikis *et al.*, 2005). It has also recently been suggested that *ELF4* functions within the clock to repress *PRR9* and *PRR7*, as well as to repress *GI*. Hence, *ELF4* was proposed to be acting as a complex to integrate the morning and evening loops of the clock (Kolmos *et al.* 2009). Post-transcriptional modifications of genes are also believed to be very important for clock function. *ZTL* is an evening-expressed gene that was first identified as a long-period circadian clock mutant, *ztl-1*, belonging to a novel family of proteins with three domains: a LOV, six Kelch and an F-box of proteins (Somers *et al.*, 2000). *ZTL* binds to both *TOC1* and *PRR5* and targets them for proteosomal degradation through interaction with the F-box. Moreover, the activity of *ZTL* is regulated by *GI* and *PRR3*. *PRR3* is also significant in light-mediated stabilisation of *TOC1* by competing for binding with *TOC1*, and preventing its interaction with *ZTL* at the beginning of the night (Para *et al.*, 2007). In blue light, *GI* may have a negative effect on *ZTL* degradation of *TOC1* and *PRR5* by blocking their interaction with *ZTL*. Hence, *TOC1* degradation is the result of a complex interplay among *TOC1, ZTL, GI* and *PRR3* (Kim *et al*, 2007).

Recently, Dai *et al.* (2011) reported that a *GARP* family transcription factor *BROTHER OF LUX ARRHYTHMO (BOA)* is a clock gene and part of the circadian oscillator. *BOA* activates expression of *CCA1* by directly binding to its newly identified promoter sites and forming a reciprocal transcriptional regulatory loop with it. Furthermore, the rhythms of *LHY, TOC1* and *GI* were affected by over-expression of *BOA*. The expression of *BOA* was also suggested to be regulated by *LHY, TOC1* and *GI*. Furthermore, *LIGHT-REGULATED WD1 (LWD1)* and *LWD2* encode *WD* (for Trp and Asp) containing proteins and were previously identified as two clock proteins regulating circadian period length and photoperiodic flowering (Wu *et al.*, 2008). Double mutation in *LWD1/LWD2* displayed an early-flowering phenotype, contributed by the significant

phase shift of *CONSTANS (CO)*, and, therefore, an increased expression of *FLOWERING LOCUS T (FT)* before dusk. Moreover, the expression phase of oscillator *CCA1, LHY, TOC1*, and *ELF4* genes displayed approximately 3 hour an advanced expression phase and short period length in the lwd1/lwd2 double mutant (Wu *et al.*, 2008). However, the latest study by Wang *et al.* (2011) indicated that both *LWD1* and *PRR9* form a positive feedback loop within the *Arabidopsis* central oscillator. *LWD1* directly associates with the *PRR9* promoter to activate its expression, whereas *PRR9* indirectly activates the expression of *LWD1* (Wang *et al.*, 2011). Further genetic and biochemical research is required to identify additional central oscillator components and place them within the clock network.

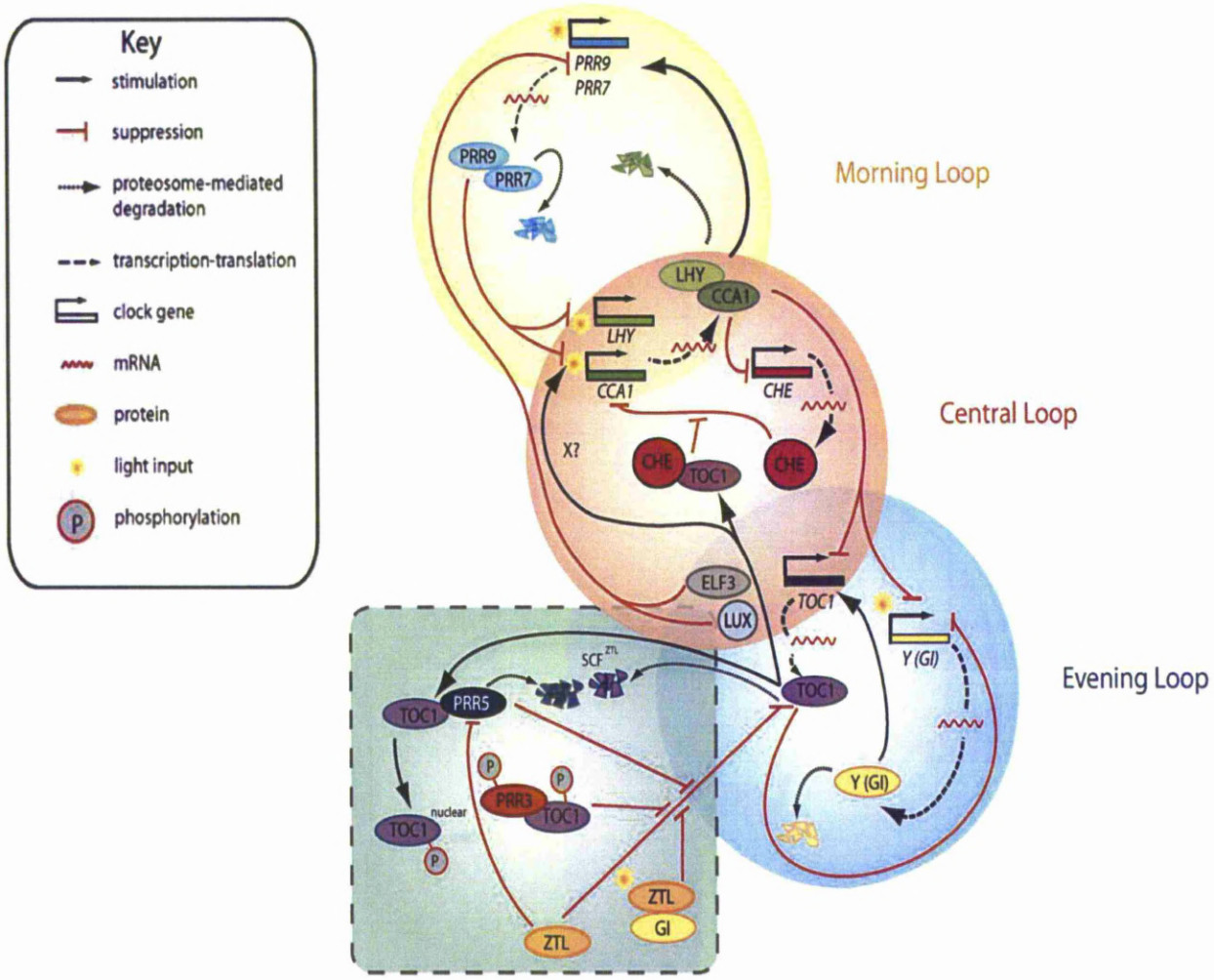


Figure 1. 2. The recent model of the *Arabidopsis* circadian clock, showing transcriptional feedback loops: the morning loop (**yellow circle**) contains the morning-phased genes *PRR7, PRR9, CCA1*, and *LHY*; the central loop (**pink circle**) includes the main clock genes LHY/CCA1, TOC1, hypothetical component X and *CHE*; and the evening loop (**blue circle**) includes the evening-phased genes *TOC1, Y/GI, ELF3* and *LUX*. Post-translational modification genes are also believed to be very essential for clock function (**green box**), which consists of *ZTL, PRR3* and *PRR5* (Haydon *et al.*, 2011).

1.3 Conservation of circadian clock genes in monocotyledons

Most of the genetic and molecular components related to comprehending the higher plant circadian clock system have been characterized in the model plant *Arabidopsis*. However, recent work has expanded these studies to cover several higher plants in order to develop our understanding of the conservation of the circadian system and its components. This analysis includes the monocot family, which comprises most of the economically important crop species such as rice, barley, maize and wheat. A high degree of conservation has been illustrated in both sequence and function of clock components using *Arabidopsis* as a framework among both *Arabidopsis* and model monocotyledons like rice (*Oryza sativa*), *Brachypodium distachyon* and *Lemna gibba* (Miwa *et al.*, 2006; Murakami *et al.*, 2006; Higgins *et al.*, 2010a). This is important, as the circadian clock is considered to be important in the regulation of key agricultural traits such as nitrate utilisation, flowering time, water use efficiency and yield (Dodd *et al.*, 2005; Gutlierez *et al.*, 2008). For this reason, plant breeders might use the circadian clock as a selectable marker for increasing yield and improving survival of crop species, or might use circadian assays to check that new varieties do not have altered clock function.

1.3.1. The PRR family

In *Arabidopsis*, the family of pseudo response regulator proteins (*PRRs*) consists of five members; all of them have been associated with the central oscillator of the plant circadian clock (Matsushika *et al.*, 2000). In addition, several *PRRs* (*PRR5, PRR7* and *PRR9*) are suggested to be involved in the regulators of photoperiod pathway and light sensitivity (Matsushika *et al.*, 2007; Nakamichi *et al.*, 2005). All *PRRs* proteins contain

two motifs that based on conservation, are thought to be important for their function. These are the pseudo receiver domain at the N terminus and the CCT domain at the C terminus (Matsushika *et al.*, 2000). A set of rice genes encoding highly homologous circadian associated *PRR* proteins has been identified and designated as *OsPRR1* analogous to *AtTOC1*, *OsPRR37* and *OsPRR73* analogous to *AtPRR3* and *AtPRR7*, and *OsPRR59* and *OsPRR95* analogous to *AtPRR5* and *AtPRR9* (Murakami *et al.*, 2003; Murakami *et al.*, 2007; Takata *et al.*, 2010). Furthermore, they are expressed in a diurnal and sequential manner from dawn to dusk in the order *OsPRR37/OsPRR73* and then *OsPRR59/OsPRR95*, followed by *OsTOC1 (OsPRR1)*. This expression pattern is similar to that of *At PRR* counterparts *(AtPRR9/ AtPRR7/AtPRR5/AtPRR3/AtTOC1)* (Murakami *et al.*, 2003; Takata *et al.*, 2010).

In 2007, Serikawa *et al.* recommended that the long day plant *Lemna gibba,* which is commonly known as duckweed, could provide a good model for studying the circadian clock in monocot species. Three *PRR* counterparts were highly conserved and pseudo receiver and the CCT domains were found; however, the clades corresponding to *AtPRR3/AtPRR7* and *AtPRR5/AtPRR3* have not been identified (Miwa *et al.*, 2006). Molecular phylogenetic analysis of PRR genes identified that two of the *Lemna* PRR homologs were similar to *OsPRR59* and *OsPRR95*, which belong to the same clade as *AtPRR5/AtPRR9* on the phylogenetic tree (Miwa *et al.*, 2006). Consequently, the sequences were designated *LgPRR59* and *LgPRR95* according to the rice, while the other homolog was nominated as *LgPRR37*, even without identification of which sequence corresponded to *OsPRR37/OsPRR73* exactly. Moreover, there was no homolog for *TOC1* (*PRR1*) which, together with *AtCCA1/LHY*, make up the *Arabidopsis* central loop; this suggests the possibility that another *Lemna* clock gene has acquired this role (Miwa *et al.*,

2006). However, to date there is no complete genome sequence of *Lemna* and upon completion we may have a better view of which of the components are missing.

In maize (*Zea mays*), a homolog of *At TOC1* has also been isolated and suggested to be a core component of the central oscillator. Quantitative real-time PCR (qPCR) analysis of *ZmTOC1* showed transcripts exhibited diurnal fluctuations in expression that peaked 12 h after dawn on long days and at 10 h after dawn on short days (Wang *et al.*, 2011). In addition, *TOC1* has been highly conserved in barley with high similarity to *AtTOC1* and *OsTOC1* sequences on the PRR phylogenetic tree. The *HvTOC1* has six blocks of amino acids including the PRR and CCT domain (C1 and C2), while the conserved blocks (C3-C6) amino acids have an unknown function (Cotter, 2010). Conservation of the function of this gene will be further investigated in this thesis.

Nakamichi *et al.* (2005a) and Turner *et al.* (2005) found that barley *PHOTOPERIOD-H1* (*PPDH1*) contains both PRR and CCT domains with sequences most closely related to *OsPRR37* and *AtPRR7*, which are key components in the morning loop and play a role in the flowering pathway. Mutation in the CCT region of the *HvPPDH1* gene *(ppd-H1* mutant) displayed a late flowering phenotype in LD but with no effect in SD. This *ppd-H1* mutant also delayed the expression of the two *HvCO* genes so that their expression peaked in the dark, leading to a decrease in *FT* expression consistent with the late flowering phenotype (Turner *et al.*, 2005). *HvPPDH1* oscillates in a circadian manner with transcription peaks in the subjective evening, which suggests that *HvPPDH1* is not analogous to *AtPRR7* but to *AtPRR3* on a phylogenetic tree (Cotter, 2010). Transcripts of *AtPRR3* also peaked in the evening and were illustrated to be involved in the flowering pathway, which is similar to *HvPPDH1* (Para *et al.*, 2007).

Therefore, *HvPPDH1* and *HvTOC1* are suggested to be members of the barley PRR family and both of them displayed circadian rhythms in constant light with evening phase peaks (Cotter, 2010). Further investigation to confirm whether *ppd-H1* mutant is clock mutant will be conducted in this thesis.

1.3.2. CCA1/LHY

The two partially redundant single MYB domain transcription factors in *Arabidopsis, CCA1* and *LHY,* are an essential part of the central feedback loop, with peak expression just before dawn (Mizoguchi *et al.*, 2002). Comprehensive analysis in the rice databases identified only a single *AtCCA1/LHY* counterpart with high conservation of the MYB domain at the N terminal; this was named *OsCCA1* (also called *OsLHY*) (Murakami *et al.*, 2006). The expression of *OsCCA1* oscillated under diurnal conditions and sustained rhythms in constant light with peaks at subjective morning that were similar to their *Arabidopsis* homolog expression patterns (Murakami *et al.*, 2006). The circadian rhythm in rice calli over-expression *OsCCA1- LUC* appeared to be almost normal with no associated phenotypes (Ogiso *et al.*, 2010). Over-expression of *OsCCA1* in *Arabidopsis* plants led to late flowering phenotypes and hypocotyl phenotypes similar to the *AtCCA1/LHY* counterpart (Murakami *et al.*, 2007a). Moreover, two copies of *CCA1/LHY* have been identified in *Lemna* plant with high sequence similarities to *AtCCA1/LHY* and *OsCCA1* homologs, designated *LgLHYH1* and *LgLHYH2* (Miwa *et al.*, 2006). This may be due to a recent duplication of the monocot *LHY* gene. The bioluminescence rhythms of the *AtCCA1:luc* and *AtTOC1:luc* reporter genes were severely dampened in *LgLHYH1* and *LgLHYH2* over-expression plants (Miwa *et al.*, 2006; Serikawa *et al.*, 2008). A *LgLHYH1* RNAi construct affected the bioluminescence rhythms of the *AtCCA1:luc* and

AtTOC1:luc reporter genes, and displayed short-period length for the *AtCCA1:luc* and a phase advance of 2 hrs with a period similar to the wild type for the *AtTOC1:luc* (Serikawa *et al.*, 2008). On the other hand, the *LgLHYH2* RNAi construct did not influence the rhythmic activity of either reporter genes, which showed similar bioluminescence rhythms to the control; this suggests that *LgLHYH1* is more involved in the generation of circadian oscillations than *LgLHYH2* (Serikawa *et al.*, 2008).

Similarly, it appears that, in *Brachypodium,* barley and maize, only a single copy of CCA1/*LHY* exists with highly conservation of the MYB domain similar to *AtCCA1/LHY* and *OsCCA1* (Cotter, 2010; Higgins *et al.*, 2010a; Wang *et al.*, 2011). Transgenic *Arabidopsis* plants over-expressing *ZmCCA1* consistently displayed the late flowering phenotype and elongated hypocotyls and transcript levels of *AtGI, AtCO* and *AtFT* were negatively affected (Wang *et al.*, 2011). Similarly over-expression of *HvCCA1* in *Arabidopsis* plants causes arrhythmic on leaf movement and delayed fluorescence with phenotypes of elongated hypocotyls and delayed flowering. Over-expression of *HvCCA1* in barley plants also severely perturbed transcript abundance of the central clock genes. The transcripts of *HvPPDH1* and *HvGI* were re-phased from the subjective evening to the subjective morning in constant light conditions and causing arrhythmic of *HvTOC1* transcript (Cotter, 2010).

1.3.3. GI

In *Arabidopsis, GI is* a nuclear protein involved in several distinct pathways including phytochrome signalling, generating clock rhythms, promoting flowering time, starch accumulation and temperature compensation (Fowler *et al.*, 1999; Gould *et al.*, 2006; Mizoguchi *et al.*, 2005). Because *GI* is involved in flowering time regulation,

characterisation and functional analysis of this gene has attracted many plant researchers, especially in crop species such as rice. The rice ortholog of *GI* (*OsGI*) has been isolated and is considered to act between the circadian oscillator and *Hd1 Hd3a* photoperiod pathway (homolog of *AtCO* and *AtFT*, respectively). The diurnal expression of the *OsGI* messenger RNA was regulated by the circadian clock and was very similar to that of *AtGI* under both SD and LD conditions (Izawa *et al.*, 2003). Over-expression of *OsGI* in transgenic rice increased Hd1 and reduced Hd3a expression leading to late flowering under both SD and LD. Loss of function of *OsGI-RNAi* resulted in an early flowering phenotype under LD conditions, which is opposite to the *Arabidopsis gi* mutants, and a late flowering phenotype under SD conditions comparative to the wild type (Hayama *et al.*, 2003). On the other hand, Izawa *et al.* (2011) pointed out that null mutation in *OsGI* was not greatly effected in the control of the flowering time of rice but controlled nearly 75% of the diurnal rhythm phases of global gene expression in the field. Loss of function of *OsGI* in the *osgi-1* null allele did not severely affect the rhythmic transcription patterns of *OsLHY, PRR73*and *PRR37* but it was required to maintain the rhythms of *PRR1*, *PRR59* and *PRR95*. Therefore, it has been suggested that *OsGI* may be a member of a sub-loop in the rice circadian clock (Izawa *et al.*, 2011).

A single homolog of *GI* (*LgGI*) was isolated from *Lemna* with 50% identity to the *AtGI* sequence (Miwa *et al.*, 2006). The *AtGI* gene displays light-stimulated diurnal rhythmic expression with a peak at around dusk (Fowler *et al.* 1999). *LgGI* exhibits a similar expression pattern to the *AtGI* homolog but it appears to be critical for maintenance of rhythms in *Lemna* (Miwa *et al.*, 2006; Serikawa *et al.*, 2008). The rhythms of both reporter genes, *AtCCA1:luc* and *AtTOC1:luc*, were dampened in over-expressing *LgGI* plants, while the rhythms of both reporters were completely abolished in the loss of function in the *LgGI* plants. Consequently, *GI* was suggested to have a more

vital role in the *Lemna* clock compared to the *AtGI* (Serikawa *et al.*, 2008). Additionally, a homolog of *GI (BdGI)* was isolated from *Brachypodium* with approximately 70% sequence identity to the *AtGI, OsGI* and *HvGI.* Similar to the *AtGI,* the *BdGI* gene was regulated by the circadian clock and was light-induced. Over-expression of the *BdGI* gene in the *Arabidopsis gi-2* mutant fully rescued the late flowering phenotype in this mutant, demonstrating that *BdGI* also plays a role in promoting flowering time (Hong *et al.*, 2010). In barley, genetic mapping showed that *GI* was found as a single copy gene (*HvGI*) and shared an amino acid identity of 79% with the *AtGI* and 94% with the *OsGI* (Dunfort *et al.*, 2005). *HvGI* transcript peaks in the evening under constant light conditions which matches the peak expression of *AtGI,* so it is likely to be involved in the evening loop of the barley circadian clock (Cotter, 2010). The function of the *HvGI* gene will be addressed further in this thesis.

1.4 Regulation of plant growth

During the plant life cycle, many biological aspects and developmental processes are regulated by the circadian clock. At the initial stage of plant growth, the circadian system is involved in the control of seed germination. Circadian rhythm in gas exchange has also been observed in *Allium cepa* seeds (Bryant, 1972). Furthermore, the imbibition (absorbance of water) in the *Arabidopsis* seeds was found to be synchronised with circadian controlled gene expression (Zhong *et al.*, 1998). *Arabidopsis* seedlings also have the ability to set the phase of clock gene expression (*LHY* , *TOC1, GI, ZTL, PRR7,* and *PRR9*) within 2 days of imbibition without any entraining by light or temperature, and mutants carrying loss of function alleles of these genes fail to initiate rhythms during the first 2 days after imbibition (Salome *et al.*, 2008). Penfield and Hall (2009) reported

that natural circadian clock gene function is crucial for the response to signalling seed dormancy release, and mutations in the clock genes *LHY*, *CCA1*, and *GI* cause germination defects in response to low temperature. It is also believed that the circadian clock is involved in regulation of chloroplast development in *Arabidopsis* seedlings though regulation of expression of both *PHYTOCHROME INTERACTING FACTOR 1(PIF1)* and 3 *(PIF3)* genes (Stephenson *et al.*, 2009). Moreover, a range of *PRR* clock mutants, including *TOC1*, failed to green normally after exposure to white light (Kato *et al.*, 2007). Fukushima et al (2009) also demonstrated that *PRR9*, 7 and *5* negatively regulate the biosynthetic pathways of chlorophyll, carotenoid and abscisic acid.

Shortly after germination, the growths of primary roots of *Arabidopsis* seedlings also exhibit a rhythm (Yazdanbakhsh and Fisahn 2009, 2010). The average daily rate of growth is raised in longer light periods or by addition of sugars. This oscillation continues in constant light and is strongly modified in clock mutants. The clock involved in primary root extension growth depends on *CCA1/LHY*; they are essential to set a proper rate of starch degradation and sustain a provider of carbon to support growth through to dawn, whereas *ELF3* acts to decline growth in the light period and promote growth in the night (Yazdanbakhsh *et al.*, 2011). Moreover, hypocotyl elongation is also controlled by the plant circadian system interacting with environmental signals (light and temperature). A clear circadian oscillation of hypocotyl elongation in constant light conditions has been detected in *Arabidopsis* seedlings with rapid hypocotyl growth occurring around subjective dusk and arrested growth near subjective dawn (Dowson-Day *et al.*, 1999). *Arabidopsis* plants that are grown under short days conditions also display maximal hypocotyl elongation in the morning with little or no growth occurring in the evening (Nozue *et al.*, 2007). Mutations in several clock-related genes (such as *CCA1/LHY,*

TOC1, GI, ZTL, FKF1, ELF3 and *ELF4)* also caused hypocotyl length alteration (Ni, 2005). Light and the circadian clock cooperate to allow hypocotyl growth to peak at dawn under diurnal cycles. Hypocotyl growth initiation before dawn is due to clock-activated transcripts of two basic helix-loop-helix (bHLH) transcription factors, *PHYTOCHROME INTERACTING FACTOR4 (PIF4)* and 5 *(PIF5)*, especially under SDs, and their proteins' abundance is degraded by light in the morning, hence growth ceases (Nozue *et al.*, 2007). Although the downstream pathways are involved in regulation of *PIF4* and *PIF5*, transcription factors by light and clock systems have not been identified yet; it has been proposed that the clock transcription factor CCA1 induced expression of both the *PIF4* and *PIF5* genes (Nozue *et al.*, 2007). A recent study has proposed that the circadian clocks control diurnal hypocotyls' growth through regulating the *ELF4–ELF3–LUX* multi-protein complex that represses the expression of *PIF4* and *PIF5* in the early evening. *ELF4* and *LUX* directly regulate plant growth while *ELF3* is necessary to form a complex between *ELF4* and *LUX.* The *ELF4–ELF3–LUX* protein complex peaks at dusk and is regulated by light (Nusinow *et al.*, 2011). *ELF4, ELF3* and *LUX* genes are believed to be part of the evening loop in the *Arabidopsis* oscillator and to act as transcription repressors of *PRR9* (Dixon *et al.*, 2011; Helfer *et al.*, 2011). It has also been suggested that *PIF4* and *PIF5* transcripts are degraded upon interaction with phyB via an active phytochrome binding (APB) domain (Lorrain *et al.*, 2008). Rhythmic leaf growth patterns in both *Arabidopsis* and *Ricinus communis* are controlled by the circadian clock, with rhythmicity persisting even under constant light conditions (Poire *et al.*, 2010).

Another way that the clock synchronises plant growth is by regulating expression of plant hormone pathways, including auxin and gibberellic acid (GA) and cytokinins. These play major roles in diurnal growth control and development. In plants grown in short days, differential genes regulated by auxin and *GA* are likely to exhibit the highest

expression at dawn, coincident with the time of maximal hypocotyl growth (Michael *et al.*, 2008). The gibberellin (GA) signalling pathway is gated by the circadian clock through transcriptional regulation of the GA receptors, *GIBBERELLIN INSENSITIVE DWARF1* (*GID1a* and *GID1b*) under short days conditions, both *GID1a* and *GID1b* repressors oscillate in a clock-dependent manner with peak expression at dusk, resulting in higher stability of DELLA proteins during daytime and lower DELLA proteins at the end of the night (Arana *et al.*, 2011). DELLA proteins repress hypocotyl growth by inhibiting PIF4 transcriptional activity via binding to the DNA-recognition domain of this factor (de Lucas *et al.*, 2008). Many of the genes involved in auxin signalling are also clock-regulated with peak expression in the night and a trough during the subjective day (Covington and Harmer, 2007; Rawat *et al.*, 2009). It has been proposed that the clock regulates the auxin level via MYB transcription factor *REVEILLE1* (*RVE1*), a *CCA1* homolog and clock output gene; this gene has been implicated in the auxin-mediated control of hypocotyl elongation. *RVE1* positively regulates the expression of the auxin biosynthetic gene *YUCCA8 (YUC8),* promoting free auxin production during the day but having no effect during the night (Rawat *et al.*, 2009). Furthermore, *PIF4* and *PIF5* are suggested to be modulators in the auxin-related pathway and control expression both of *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN2 (ATHB2)* and auxin-inducible *IAA29* with a peak at dawn specifically in short days (Nouzue *et al.*, 2011; Kunihiro *et al.*, 2011). *ARABIDOPSIS RESPONSE REGULATOR4 (ARR4)* seems to be acting as a mediator in cytokinin signalling and circadian light input via interacting with phyB. Loss of function of *ARR4* lengthens the period of the clock even in the absence of light; the circadian defects in the *arr4* mutant appear to be unrelated to cytokinin, as exogenous cytokinin altered the phase but not the period of the clock (Salome *et al.*, 2006).

The circadian clock is likely to be involved in regulation both of the cellular processes, photosynthesis and carbon fixation occurring at a specific time of day. In *Arabidopsis*, many of the key genes that participate in the light harvesting complex and PSI and II are under circadian control at the level of the associated steady-state transcript abundance (Harmer et al., 2000). The rhythms of photosynthesis gene expression appear to be associated with circadian rhythms of both stomata opening and CO2 assimilation (Salome *et al.*, 2002). In addition, observation of chloroplast gene expression in *Chlamydomonas reinhardtii* using *psbD-lucCP* bioluminescence reporter indicated that the period length of the chloroplast rhythm was associated with the nucleus-encoded circadian oscillator (Matsuo *et al.*, 2006). What is more, *Phosphoenolpyruvate carboxylase (PEPC)* activity of *Crassulacean acid metabolism (CAM)* plants is regulated by circadian clock via *PEPC* kinase (Hartwell et al., 2002).

The circadian clock had the ability to anticipate light dark cycles, and enhance plant fitness and metabolism. According to Dodd *et al.* (2005), plants with an endogenous period matched to the environment confer an adaptive advantage. A set of competition experiments using short period (*toc1-1*) and long period (*ztl-1*) mutants confirms that, when the photoperiods correspond with their endogenous circadian systems, plants produce a larger biomass, contain more chlorophyll and fix more carbon than in a 12 h light dark cycle (T24). Whereas, the arrhythmic *CCA1-ox* mutant has decreased water use efficiency, dry weight and photosynthetic CO2 fixation compared to wild-type plants growing under T24 (Dodd *et al.*, 2005). Starch degradation in *Arabidopsis* plants appears to be under circadian clock control to maintain carbohydrate availability until the next anticipated dawn, and this control is essential for preserving plant productivity (Graf *et al.* 2010). Starch of *Arabidopsis* plants growing in abnormal day lengths (T28 or T17) was exhausted about 24 h after the last dawn, irrespective of the actual dawn.

Consequently, starch was exhausted about 4 h before the actual dawn in T28. Additionally, the *lhy/cca1* double mutant exhausted its starch about 20 h into the diurnal cycle (Graf *et al.* 2010).

1.5 Photoperiodism and control of flowering time

The circadian clock can measure day length, thus regulate flowering time (Niwa *et al.,* 2007). Recent intensive studies on the model plant *Arabidopsis thaliana* have begun to shed light on the molecular mechanisms of the photoperiod pathway and have tried to identify the roles the circadian clock plays in controlling it. It is believed that the main factors linking clock function and floral induction are flowering integrator genes, *CONSTANS (CO)*,members of the CCT protein family, which encode a nuclear-localised zinc finger-containing protein, which was recently proposed to serve as a component of a DNA-binding transcription factor (generally called the HAP complex) (Wenkel *et al.* 2006); and *FLOWERING LOCUS T (FT)* that encodes a small approximately 20-kD protein with homology to the Raf kinase inhibitor protein of animals, and acts in the shoot apical meristem to induce the switch from vegetative to floral meristem through the interaction with the bZIP transcription factor *FLOWERING LOCUS D (FD)* (Abe *et al.*, 2005; Fujiwara *et al.,* 2005; Cockram *et al.,* 2007; Wigge *et al.*, 2005). This is dependent on *CO* protein, which acts as an activator in a light-dependent manner (Nakamichi *et al.*, 2007; Niwa *et al.*, 2007).

Recently, it has been reported that a diverse range of regulators was involved in strictly regulating expression of *FT* (Figure1.3), including *FLOWERING LOCUS C (FLC), SHORT VEGETATIVE PHASE (SVP), TERMINAL FLOWER2 (TFL2), EARLY BOLTING IN SHORT DAYS (EBS), TEMPRANILLO (TEM1* and *TEM2*), *CURLY LEAF*

(CLF) and *FERTILIZATION INDEPENDENT ENDOSPERM (FIE)* (Jiang *et al.*, 2008; Li *et al.*, 2008; Searle *et al.*, 2006; Takada and Goto, 2003). The MADS box transcription factor *FLC* acts as a floral repressor and mediates the autonomous and vernalization pathways, and operates in a repressor complex together with *SVP* protein to inhibit expression of *FT* by binding directly into the CArG-box DNA motifs in the first intron of the *FT* gene (Li *et al.*, 2008; Searle *et al.*, 2006). The transcription of *FLC* during vernalization (exposure to low temperatures soon after germination) is promoted by the gene *FRIGIDA* (*FRI*) and plant homeodomain (PHD) finger proteins *VERNALIZATION INSENSITIVE 3 (VIN3),* plant-specific DNA binding proteins *VERNALIZATION 1 (VRN1),* and SU(Z)12-like polycomb-group protein *VERNALIZATION 2 (VRN2)* (Amasino, 2004; Kim *et al.*, 2009). *FRI* activates *FLC* expression to delay flowering through interaction with the histone methyl transferase *EARLY FLOWERING IN SHORT DAYS (EFS), FRIGIDA LIKE1 (FRL1), FRIGIDA ESSENTIAL1 (FES1), SUPPRESSOR of FRIGIDA4 (SUF4)* and *FLC EXPRESSOR (FLX)* to form the *FRI* transcription activator complex *(FRI-C),* which results in the modification of *FLC* chromatin (Kim *et al.*, 2009; Ko *et al.*, 2010; Choi *et al.*, 2011). Transcriptional repression of *FLC* was regulated by *VRN1* and *VRN2* by association with a region near the 5#-end of intron 1 of *FLC* dependent on the presence of *VIN3* that binds to the chromatin of the *FLC* locus and alters its structure (Sung and Amasino, 2004; De Lucia *et al.*, 2008; Bond *et al.*, 2009). Moreover, the chromatin-associated proteins, *TLF2* and *EBS*, suppress *FT* transcription by direct binding in *FT* chromatin (Takada and Goto, 2003). The subunits of *Arabidopsis* Polycomb Repressive Complex2, *CLF* and *FIE*, are strongly suggested to act as *FT* repressors during vegetative development (Jiang *et al.*, 2008). Both of the RAV subfamily transcription factors, *TEM1* and *TEM2*, also function as repressors of FT expression via direct binding to its 5'UTR region (Castillejo and Pelaz, 2008).

Furthermore, a homolog of *HETEROCHROMATIN PROTEIN1 (HP1),* the polycomb protein *TERMINAL FLOWER2* (TFL2*)*, appears to be playing an important role in preventing activation of *FT* at the shoot apex (Takada and Goto, 2003).

It seems that *FT* promotes the transition to flowering by activating other floral integrators, SUPPRESSOR *OF OVEREXPRESSION OF CONSTANS 1 (SOC1), AGAMOUSLIKE 24 (AGL24), LEAFY (LFY), FRUITFULL (FUL), CAULIFLOWER (CAL)* and *APETALA1 (AP1)* (Adrian *et al.*, 2009). In the apical meristem, FT induces expression of both members of the MADS-box family, *SOC1* and *AGL24*, which are also believed to physically interact and positively regulate each other by direct binding to their respective promoters; and the complex of the two proteins directly activates the plant-specific transcription factor *LFY* (Lee *et al.*, 2008; Adrian *et al.*, 2009). *LFY* was fed forward to activate *AP1* directly by binding on its promoter, and its redundant homolog *CAL* in the floral meristem, which itself positively feeds back on *LFY* and directly represses *SOC1/AGL24* in the floral meristem. Therefore, *LFY* and *AP1/CAL* support each other's expression and stabilise the floral development programme (Adrian *et al.*, 2009; Benlloch *et al.*, 2011). *FUL* is also proposed to be involved in flower development and associated with AP1 regulation but the mechanism has not yet been elucidated (Adrian *et al.*, 2009). In contrast, *TERMINAL FLOWER 1 (TFL1)* seems to interact with *FD* in the floral meristem to inhibit floral initiation through suppression of the expression of *AP1* and *LFY* (Hanano and Goto, 2011). *TFL1* also belongs to the same CETS family as *FT*, and has homology with a phosphatidylethanolamine binding protein (PEBP) (Pnueli *et al.*, 2001). Both *FT* and *TFL1* proteins share nearly 71% similarity with amino acid residues but are functionally antagonistic (Hanzawa *et al.*, 2005). Gibberellins were also believed to be involved in *Arabidopsis* promotion of flowering by induction of the floral transition at the shoot apex, particularly under SD (Gottgens and Hedden, 2009).

Expression levels of both *CO* and *FT* are under circadian clock control possibly via *GI, FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1), RED AND FARRED INSENSITIVE 2 (RFI2)* and *CYCLING DOF FACTOR1 (CDF1)* (Nakamichi *et al.*, 2007). *GI* acts as a transcriptional activator for *CO*, while *CDF1* encodes a Dof-domain containing DNA-binding protein and serves as a transcriptional repressor via directly binding to the *CO* promoter (Dunford *et al.*, 2005; Mizoguchi *et al.*, 2005; Nakamichi *et al.*, 2007). *FKF1* protein is proposed to form a blue-light-dependent complex with *GI*, which promotes the degradation of the *CDF1* protein in late daytime (evening manner) by functioning as a component of E3 ligase (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). The *RFI2* gene is believed to be acting in both the phyA and phyB signalling pathways, and encodes a protein contains a RING-finger domain with transcript peaking at the end of the night under both LD and SD similar to that of *CO*. This suggests that it is involved in *CO* protein degradation by repressing the expression of *CO*, whereas, *GI* may maintain the proper expression of *RFI2* through its positive action on the circadian clock (Chen *et al.*, 2006). Recent studies have demonstrated that *GI* can regulate *FT* expression independently of *CO* through regulated miR172, a small RNA, or by binding to three *FT* repressors: *SVP, TEM1* and *TEM2. GI* is also capable of directly activating FT expression by binding to its promoter region, which is near the *SVP* binding sites (Jung *et al.*, 2007; Sawa and Kay, 2011). The main question arising is how the clock associated components (*CCA1/LHY* and *TOC1*) are co-ordinately linked to the CO–FT pathway. In this respect, a close link between the central clock circuitry and the downstream CO–FT photoperiodic pathways has been investigated previously (Mizoguchi *et al.*, 2005). It was suggested that *CCA1/LHY* act as a repressor to *CO* and *FT* genes through negatively regulating the transcription of *GI*, dependent on the *TOC1* gene, which acts as an activator (Mizoguchi *et al.*, 2005; Niwa *et al.*, 2007; Sawa and Kay, 2011). Furthermore, *CCA1* and *LHY*

accelerated flowering in constant light conditions by promoting *FT* expression through reducing the abundance of *SVP* (Fujiwara *et al.*, 2008), whereas *PRR9, PRR7* and *PRR5* act as activators to the *CO* gene by repressing *CDF1* (Turner *et al.*, 2005; Nakamichi *et al.*, 2007).

It has been demonstrated in *Arabidopsis* that disruption of circadian function causes altered flowering times and reduced plant performance. For instance, loss of function of the main oscillator components *LHY*, *CCA1* or *TOC1* causes circadian rhythms to cycle with a shorter period and the plants flower earlier than the wild type under short day conditions (Mizoguchi *et al.*, 2002; Niwa *et al.*, 2007). The *GI* loss of function mutant exhibits an extremely late flowering phenotype in long day conditions; it also plays a fundamental role in the highly conserved flowering pathway (Fujiwara *et al.*, 2005). Moreover, *Arabidopsis* plants with a functional clock have a substantial advantage over plants defective in clock function (Dodd *et al.*, 2005). Because flowering time has been an important trait for improving crop productivity and adaptation, differences in flowering time have been selected by plant breeders for many years, in order to increase yield and extend agriculture flexibility (Cockram *et al.*, 2007). As the circadian clock is involved in CO-FT photoperiodic pathway regulation, characterisation of the main clock genes in crop species is important. What is the key to altering flowering time without altering the performance benefits of having a robust and accurate circadian clock?

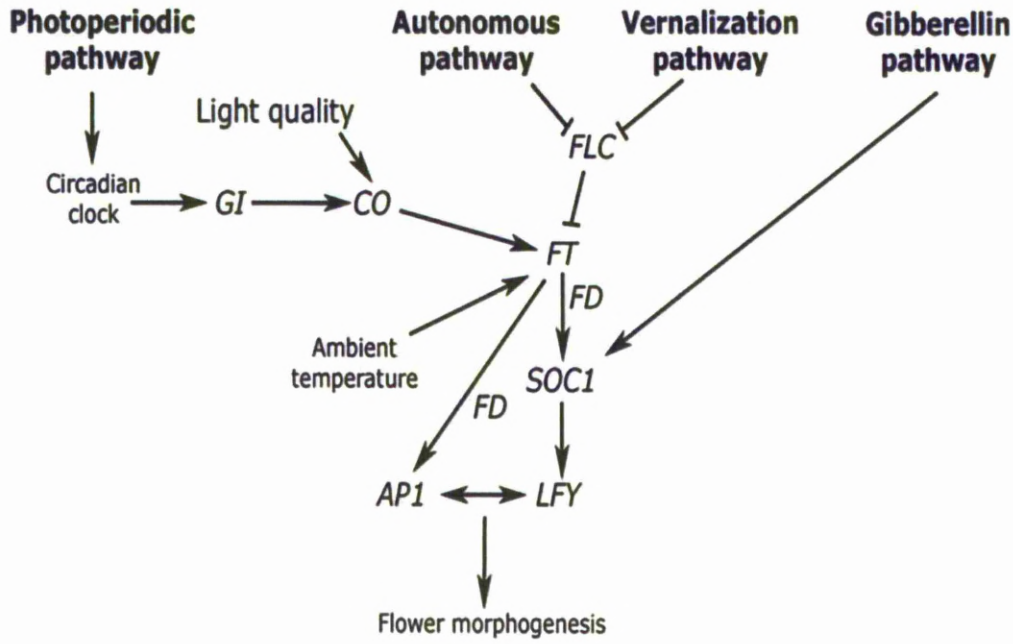


Figure 1.3. Simplified schematic illustrating flowering time gene interactions in *Arabidopsis thaliana*. The autonomous pathway consists of *PRR9, PRR7, PRR5, FKF1, CDF1* and *CO*. The vernalization pathway is mediated in part by *VRN1, VRN2, VIN3, FRI*, and *FLC*. The photoperiod pathway includs genes *CCA1/LHY, TOC1* and *GI*. The gibberellin pathway promotes flowering time. These inputs seem to be integrated by *FT* and *SOC1*, which in turn proceed via *AP1* and *LFY* to regulate flowering (Corbesier and Coupland, 2006).

1.6 Aim of the study

This study aims to understand the role that the circadian clock plays in regulating growth and development of crop plants, especially barley. This information could be exploited to select plants having adaptive traits under environmental cues, which could lead to increased productivity of current and future crops. The main crop species selected for this study is barley (*Hordeum vulgare* L.). Barley is ranked fourth most important cereal crop globally after wheat, rice and maize. It is also an excellent model for cereal crop plant improvement and a major target in biotechnology plant research, with a large amount of genomic resources available on public databases. Another advantage of studying barley is that it is a self-pollinating diploid (2n = 2x = 14) plant with a large genome size - 5000 Mb. 50-60% of the genome consists of repeated sequences (Suchankova *et al.,* 2006). The diploid nature makes barley more suitable for plant and genetic studies compared with hexaploid crops such as wheat (Varshney *et al.,* 2004; Suchankova *et al.,* 2006).

This research study was divided into four distinct sections. The first section of this work looked at expression of main barley clock genes. These genes were knocked down using RNA-interference (RNAi) techniques to discover what effects silencing these genes had on the plant's performance and to investigate if there was a similarity to losing the functions of these genes in *Arabidopsis* plants.

In the second section, the barley *GIGANTEA* gene was over-expressed *Arabidopsis* plant to examine whether the biological function of the *GI* gene exhibited high conservation through plant species. In the third section, the aim was to quantification of the importance of light and temperature compensation of the circadian clock in the enhancement of growth and fitness of barley plants, and this was determined using

commercial flowering mutants. The main flowering mutant investigated was the late flowering phenotype *ppd-H1*, which is most similar overall to the *Arabidopsis PRR7* mutant. The last section of the thesis was mainly focused on development of a rapid and simple clock assay for measuring robustness and accuracy of the barley circadian clock by using delayed fluorescence.

CHAPTER 2: MATERIALS AND METHODS

2.1. Barley transformation

2.1.1 DNA amplification

The primers were designed to generate RNAi fragments to silence target barley clock genes *CCA1*, *TOC1* and *GI* from genomic DNA of barley (Cv. Golden Promises) by using the program primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3). All primers used for each PCR reaction were synthesized by Invitrogen (Table 2.1). The position of primers on barley genomic DNA is illustrated in Appendix 1.

All the PCR reactions for each gene were set up in a 25µl final volume reaction mixture. The mixture contained 2.5µl 10x PCR buffer for KOD hot start DNA polymerase, 2µl of 2 mM $MgSO_4$, 2.5µl of 0.2 mM dNTP, 0.5µl of 1 U/µl of KOD hot start DNA polymerase, 0.5µl of 50ng/ µl DNA sample, 1.5µl forward primer (10 mM stock), 1.5µl reverse primer (10 mM stock), and 15µl PCR grade water, bringing the total volume of the mixture to 25µl. Each mixture was kept on ice, vortexed briefly and centrifuged for one min at 300 rpm. The PCR mixtures were placed in an MJ Research Dyad DNA Engine Peltier thermal cycle machine. Hot start PCR was denatured with 94°C for 2 min followed by 39 cycles of 94°C for 1 min, 68°C for 30sec and 68°C for 1 min.

Table 2.1. Primers used to amplify target barley clock genes.

Name	Sequence	Length	TM
TOC1RNAi Forward	CCAGGTTAATTTCTCCGGTTCGACTGAC	28	68.81
TOC1RNAi Reverse	ATACCGATGACCACACATTCTGCATTGA	28	68.70
GI RNAi Forward	TTCACTGAAGCGATGTAAGTGGGATGC	27	69.96
GI RNAi Reverse	CTGAGAGCCTCGATAACCCCCATTTCT	27	69.91
CCA1 RNAi Forward	CAGTGCAACCAAGATGCCTA	20	59.96
CCA1 RNAi Reverse	GGACCCAGAAGATGCTACAACT	22	60.39
Hygromycin Forward	ACTCACCGCGACGTCTGTC	19	60.89
Hygromycin Reverse	GCGCGTCTGCTGCTCCAT	18	64.64
***ccdB* gene Forward**	ATGCAGTTTAAGGTTTACACC	21	65
***ccdB* gene Reverse**	TAACCTGATGTTCTGGGGAATATA	24	69
pBract207 introns sequence Forward	TGGCGATTATTGCTCAGTTG	20	58.31
pBract207 introns sequence Reverse	GGTAGAAGCAGAACTTACGTACA	23	54.20
pBract207 Forward	GTGTTACTTCGCCCAGCTTC	20	58.39
pBract207 Reverse	TGTTTGAACGATCCTGCTTG	20	58.28
HvGI-PCMD32 Forward	ATCGCAAGACCGGAACA		
HvGI-PCMD32 Reverse	CCTCTTCGCTATTACGCCAG		

2.1.2 Agarose gel electrophoresis for separation of DNA

The PCR product was analysed by a high-resolution agarose gel (0.7%) electrophoresis in 10x TAE (Tris-acetate-EDTA) buffer; 0.7g of agarose was melted in 100 ml of 1x TAE buffer to make 0.7% gel, as described by Warude *et al.* (2003). To make a final concentration of 0.5 μg/ml, Ethidium bromide was added to the melted gel, mixed thoroughly then poured into a mould with a comb. The gel was left to set at room temperature for 20 min, then the comb was gently removed and the solidified gel was placed slowly into an electrophoresis tank filled with 1 x TAE buffer. DNA samples were loaded into the wells after mixing them with 2μl of 5x DNA loading buffer. The gel was run using a voltage of 90 V/cm. The DNA fragments were visualized using UVP trans-illuminator and a thermal gel print was taken by a UP-895CE graphic printer (Sony). The DNA fragments' size were determined by using standard DNA ladder (Hyper ladder I). The correct size of products was cut out from the gel and cleaned using MinElute gel extraction kit (Qiagen, Hilden, Germany).

2.1.3 Plasmid transformation

The PCR products were cloned into PCR®8/GW/TOPO® entry vector (Figure 2.1) using pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen), following the manufacturer's procedure. The standard TOPO reaction mix was 1μl of 55ng PCR®8/GW/TOPO® construct, 2.5μl of 100ng gel extracted PCR product, 1μl salt solution, and 1.5μl of sterilised distilled water was added to bring the total volume of the mixture to 6μl. The mixture was incubated for 30 min at room temperature and then placed on ice for 2 min. The PCR®8/GW/TOPO® constructs were transferred to the *Escherichia coli* strain Exl blue by electroporation at field strength 2.5kv/cm, a capacitance of 25μF and resistance of 200 ohms in parallel with the sample. Transformed cells were spread on plates

containing LA media with 50μg/ml Kanamycin and incubated at 37°C overnight. Several colonies were selected randomly to check the presence of the right construct in *E. coli.* Parts of the selected colonies were used to run colony PCR with the primers used to amplify target DNA. The other parts of the selected colonies were incubated in a LB medium with 50μg/ml Kanamycin in 37°C overnight. After PCR confirmation, plasmid DNA was extracted from the bacteria culture by using QIAprep® miniprep kit (Qiagen). Then, the new constructs were fully sequenced to confirm no PCR errors existed and the correct fragment had been inserted.

The PCR fragments were transferred from PCR®8/GW/TOPO® entry vector to the Gateway destination vector pBract207 (Figure 2.2A and B) (http://www.bract.org) by recombinase reaction using Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen). The standard LR reaction mix was 1μl of 55ng PCR®8/GW/TOPO® construct, 1μl of 100ng PBract207 vector, 2μl of LR clonase, and 6μl of sterilised distilled water was added to bring the total volume of the mixture to 10μl. The mixture was made following the manufacturer's instructions. Then the mixture was incubated for 16 h at 25°C. At the end of the incubation time, the reaction was terminated with 1ul proteinase K, mixed and incubated again for 10 min at 37°C. New constructs were transferred to the *E.coli* strain Exl blue by electroporation, as described above. The transformed cells were plated on LA media containing 50μg/ml Kanamycin and incubated overnight at 37°C. Twelve colonies from the obtained *E. coli* were incubated on the same media and conditions. Then, the plasmid DNA was purified from the bacteria culture by using QIAprep® miniprep kit (Qiagen) and was tested by being digested by the *EcoRV* and *PvuI* restriction enzymes. For confirmation, plasmid DNA containing the newly made constructs was fully sequenced at John Innes genome laboratory sequencing service. The sample sent for

sequencing contained a 20 1µl of 100ng of newly made constructs, 20 1µl of pBract207 forward (10 mM stock) and 20 1µl of pBract207 reverse (10 mM stock) (Table 2.1). The sequence data were imported into the Generous pro 4.5.4 Software and blasted against the template sequences of *TOC1* and *GI RNAi* products (Appendix 1).

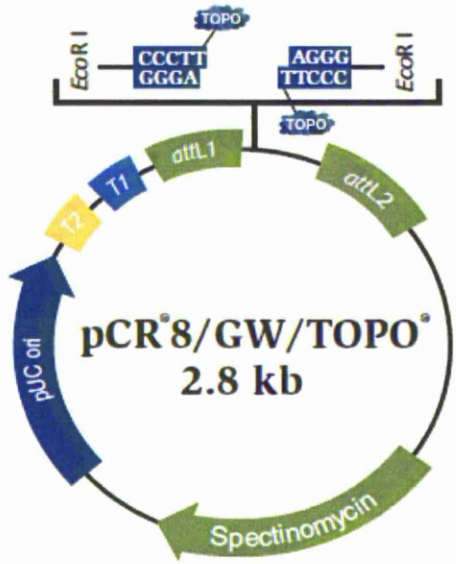


Figure 2.1. Map of pCR®8/GW/TOPO® entry vector. http://tools.invitrogen.com/content/sfs/manuals/pcr8gwtopo_man.pdf.

2.1.4 Plant material

According to Shrawat *et al.* (2006), barley seeds (*Hordeum vulgare* l. Cv. Golden Promise) were cultivated in a greenhouse at 23°C. 12-14 days after plant pollination, barley spikes were collected. At this stage, the length of the immature embryo was approximately between 1.5-2.5 mm. The awns were completely removed from the grains without damaging the seed coat and immature seeds were surface sterilised with 70% (v/v) ethanol for 1 min, followed by three washes in sterilised distilled water. This was

followed by soaking the seeds in 50% sodium hypochlorite for 4 min whilst gently shaking the container. After that, the seeds were rinsed four times in sterilised distilled water, as described by Tingay *et al* (1997).

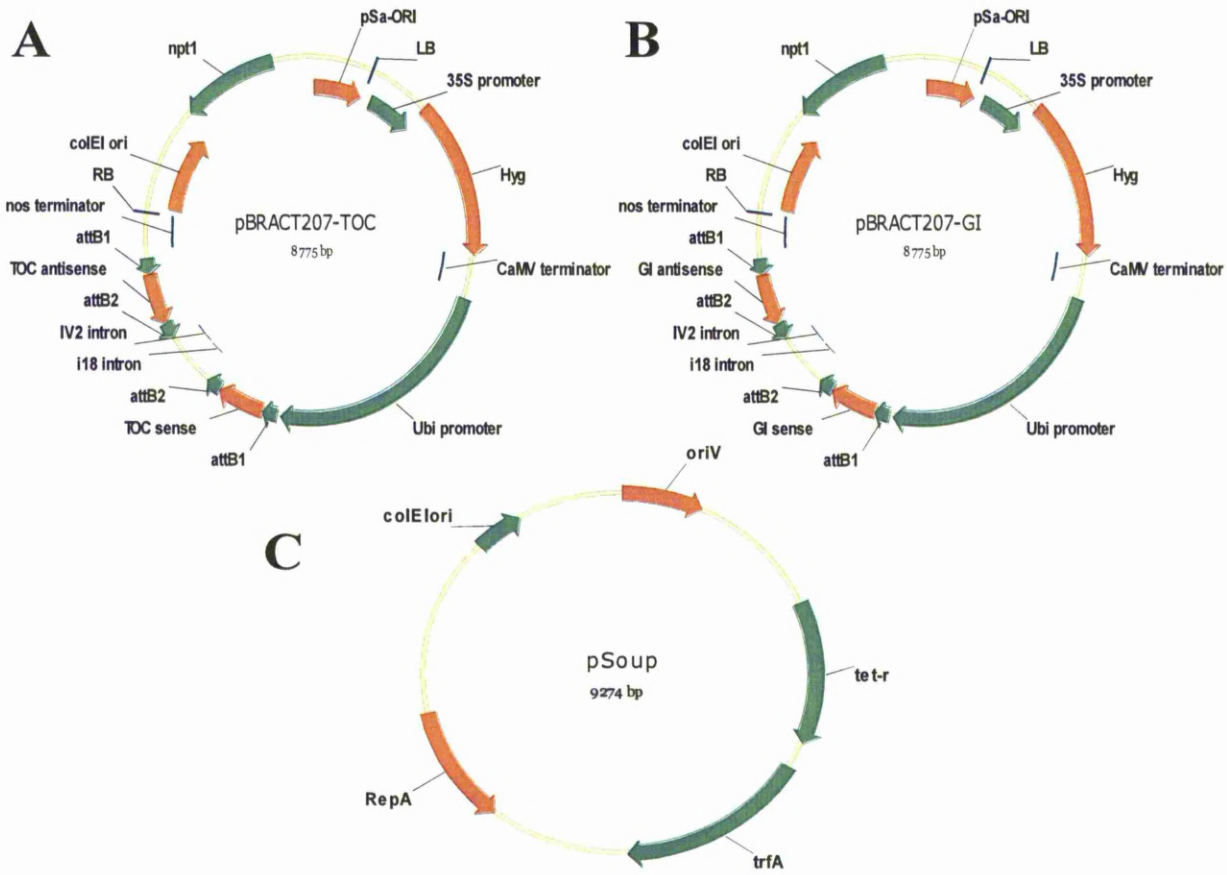


Figure 2.2. Plasmid map. (A) Map of pBract 207 plasmid cantians *TOC1* gene which are controlled by a maize ubiqitin promoter. (B) Map of pBract 207 plasmid cantians *GI* gene which are controlled by a maize ubiqitin promoter. (C) Map of the pSoup plasmid contains trans-acting replicase gene (RepA) (http://www.bract.org).

2.1.5 *Agrobacterium* strain and transformation vectors

Agrobacterium tumefaciens strain AGL1 carrying pBract207 plasmid together with helper plasmid pSoup was used. The pBract207 vector has a pGreen backbone and contains two LR cloning sites which are separated by introns at the right border. That allowed GUS genes (encoding *TOC1* or *GI*) to flank in the opposite direction in order to produce a hairpin RNA loop when the introns are spliced out. At the left border, the plasmid has a Hygromycin resistant gene driven by the cauliflower mosaic virus (*CaMV)* 35s promoter (Figures 2.2A and B) (http://www.bract.org). The helper plasmid pSoup (Figure 2.2C) provides replication functions for pBract207 (http://www.bract.org). The two plasmids pBract207 and pSoup were introduced into strain AGL1 by electroporation at field strength 2.5kv/cm, a capacitance of 25μF and resistance of 200 ohms in parallel with the sample (Weigel and Glazebrook, 2002). After that, the AGL cells were transferred immediately to a clean tube and incubated by shaking in a 500 ml LB medium for 3-4 hours at 28°C. After that, the transformed cells were plated on an LA medium with 25 μg ml^{-1} Rifampicin and 50 μg ml^{-1} Kanamycin antibiotics and incubated at 28°C for 2 nights. To examine if the AGL carried pBract207 /pSoup constructs, six clones were incubated in an LB medium with 25 μg ml^{-1} Rifampicin and 50 μg ml^{-1} Kanamycin antibiotics overnight on a rotary shaker (150 rpm) at 28°C. The pBract207 plasmid was isolated from the bacterial culture using QIAprep® miniprep kit (Qiagen) and retransferred to the *E. coli* strain Exl blue using a method similar to the one described above. Then the pBract207 plasmid was extracted from the *E. coli* culture using QIAprep® miniprep kit (Qiagen) and tested by being digested by *EcoRV* restriction enzyme. AGL1 clones carrying the plasmid were used to prepare bacterium standard inoculums.

2.1.6 Preparation of *Agrobacterium* standard inoculum

A single colony of AGL1 strain carrying pBract207 was incubated in a 10 ml LB medium, containing 25μg ml^{-1} Rifampicin and 50 μg ml^{-1} Kanamycin antibiotics, overnight on a rotary shaker (150rpm) at 28°C. A volume of 10 ml sterile 30% aqueous glycerol was added to the bacterial culture and thoroughly mixed several times by high speed vortexing. Aliquots of 500μl of standard inoculum were placed into 0.5 ml eppendorf tubes and then stored at -80°C.

2.1.7 Embryos' isolation and transformation

As described by Harwood *et al.* (2000), immature embryos were isolated from immature seeds under a dissecting microscope in sterile conditions and transferred to a callus induction medium (CI; Bartlett *et al.,* 2008). The *Agrobacterium* cell suspension was dropped onto each immature embryo by using sterile pipettes and then plates were tilted in the laminar air flow cabinet to remove any excess *Agrobacterium* cells from the embryos. To remove excess *Agrobacterium*, the immature embryos were gently dragged across the surface of the medium and transferred to fresh CI plates. Then the plates were sealed and incubated in the dark at 23-24°C and co-cultivated for three days with the scutellum side up. After the embryos were infected with *Agrobacterium*, they were transferred to a fresh CI medium containing 160 mg/l^{-1} Timentin (Duchefa) to inhibit the growth of *Agrobacterium* cells during the embryogenic callus, and 50 mg/l^{-1} Hygromycin as selective agents. The embryos were sub-cultured onto fresh CI selection medium plates every two weeks and incubated again in the dark at 23-24°C. After six weeks, the embryo-derived calluses were transferred to a transition medium (T), which also contained Hygromycin and Timentin, for two weeks at 23-24°C under low light, which was provided by covering the plates with a thin sheet of paper. At this stage, as expected,

the calluses were producing green areas. The calluses-derived small shoots were transferred to a regeneration medium (R) in deep Petri dishes, in full light with the same levels of Hygromycin and Timentin, without any growth regulators, keeping all calluses from a single embryo together. When the plantlets regenerated 2-3cm shoots, they were transferred to a glass culture tube containing a CI medium without any growth regulators but still containing the same levels of Hygromycin and Timentin. After 2-3 weeks, transformed plants that had developed a strong root system in the Hygromycin-containing medium were transferred into the soil and grown under the same conditions as the donor plants.

2.1.8 PCR analysis

To prove the presences of the Hygromycin (hpt) gene in transgenic embryos and plants transformed with *TOC1* and *GI RNAi* constructs, PCR analysis was conducted. DNA was extracted from both the embryos and plant leaves by using the Qiagen DNAsy miniprep Plant kit (Q69181) system following the manufacturer's instructions. A 917-bp fragment of the hpt gene was amplified using Hygromycin primers (Table 2.1). Reactions were composed of 5µl RED Taq® ready mix™ PCR Reaction (3 mM MgCl2), 1µl of each primer and 1µl of template DNA, and 2µl PCR Grade water, bringing the total volume of mixture to 10µl. PCR was carried out using an MJ Research Dyad DNA Engine Peltier thermal cycle machine, with the conditions 95°C for 1 minute, followed by 30 times of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and finishing with 72°C for 10 minutes. The PCR samples were separated on 0.7% agarose gels (containing 3µl of Ethidium Bromide per 100 ml) using gel electrophoresis.

In order to confirm that transformation was successful with *TOC1* and *GI RNAi* constructs, extra PCR analysis was run with primers specific (containing the inserted T-DNA sequence), *ccdB* primers and pBract207 introns sequences primers (Table 2.1). The reactions were prepared as described above. Then they were placed in a thermal cycle machine with conditions of 95°C for 1 minute, followed by 39 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The PCR samples were separated on 0.7% agarose gels, as described above.

2.1.9 *Agrobacterium* contamination test

In order to ensure that the *Agrobacterium* used to carry the silencing constructs was not contaminated with other strains of bacteria, *Agrobacterium tumefaciens* strain AGL1 carrying *TOC1* and *GI RNAi* constructs was incubated for two days at 28°C on plates containing Eosin methylene blue agar medium (37.5g agar per 1 litter DSW), and EXM medium (%yeast extract, 2% glucose, 2% $CaCO_3$, and 2% agar). After being incubated for 2 days, the plates were flooded with a shallow layer of Bencdict's reagents (17.3g Sodium citrate, 10g Sodium carbonate, 1.73g $CuSO_4.5H_2O$ in 100 ml DSW). The positive test: formation of a yellow ring of cuprous oxide around the *Agrobacterium* growth.

Moreover, 400-bp fragments of the *TOC1* and *GI* genes were amplified using specific primers (Table 2.1). Reactions were composed of 5μl RED Taq® ready mix™ PCR Reaction (3 mM $MgCl_2$), 1μl of each primer and 1μl of template DNA, and 2μl PCR Grade water, bringing the total volume of mixture to 10μl. PCR was carried out using an MJ Research Dyad DNA Engine Peltier thermal cycle machine, with the conditions of 95°C for 1 minute, followed by 30 times of 95°C for 30 seconds, 60°C for 30 seconds,

72°C for 1 minute, finishing with 72°C for 10 minutes. The PCR samples were separated on 0.7% agarose gels (containing 3µl of Ethidium Bromide per 100 ml) using gel electrophoresis.

2.2. *HvGI* over-expression in *Arabidopsis* plants

2.2.1. Plasmid transformation

The *Hv-CDNA GI* gene was transferred from pENTR™/D-TOPO® entry vector (Figure 2.3, A) to the binary vector PMDC32 carrying CaMV 2x35S promoter and Hygromycin resistant gene at the left border (Figure 2.3, B) by a recombination reaction using Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen). The standard LR reaction mix was 1µl of 55ng pENTR™/D-TOPO® construct, 1µl of 100ng PMDC32 vector, 2µl of LR clonase, and 6µl of sterilised distilled water, bringing the total volume of the mixture to 10µl. The mixture was made following the manufacturer's instructions. Then the mixture was incubated for 16 h at 25°C. At the end of the incubation period, the reaction was terminated with 1µl proteinase K, mixed and incubated for 10 min at 37°C. New constructs were transferred to the *E. coli* strain Exl blue by electroporation, as described above. The transformed cells were plated on LA media containing 50µg/ml Kanamycin and incubated overnight at 37°C. The obtained *E. coli* colonies were grown in a LB medium with 50µg/ml Kanamycin overnight at 37°C. Then the plasmid DNA was isolated from the bacterial culture by using QIAprep® miniprep kit (Qiagen) and tested by being digested by *EcoR5* and *Acc65I* restriction enzyme. For confirmation, the plasmids DNA containing the newly made construct was fully sequenced as described above using *HvGI-PCMD32* primers (Table 2.1) (The result of the sequencing can be found in Appendix 1).

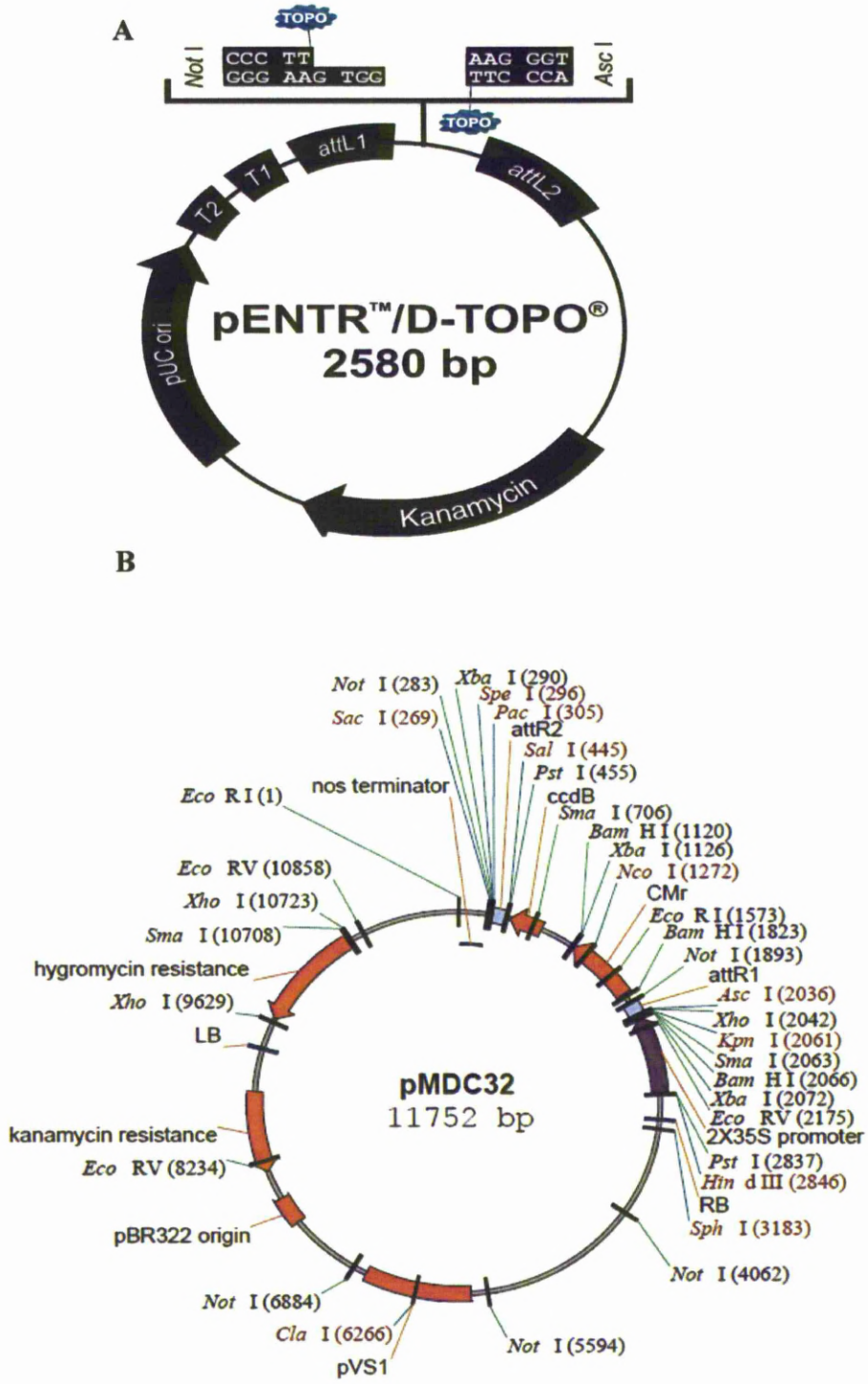


Figure 2.3. Plasmid map. **(A)** Map of pENTRTM/D-TOPO® entry vector. http://tools.invitrogen.com/content/sfs/manuals/pentr_dtopo_man.pdf. **(B)** Map of PMDC32 destination vector. http://botserv1.uzh.ch/home/grossnik/curtisvector/pMDC32.pdf.

2.2.2. Plant material

Seeds of wild-type *Arabidopsis thaliana* L. Heynh ecotype wassileskija (WS) and *gi-11* null mutant (T-DNA inserted mutant generated using the PGKB5 tagging vector) were surface sterilised in 70% ethanol immediately followed by 50% bleach with 0.01% Tween 20 for 10 min. Then the seeds were rinsed with sterile distilled water (SDW) and re-suspended in 0.1% agar. After stratification of the seeds in the dark at 4°C for three days, the seeds were finally sown onto a Murashige and skoog (MS) medium containing 1.5% agar. The seedlings were grown under controlled conditions in the plant growth room with 12:12 h light:dark cycle at 22°C for 10 days. Then, 15 seedlings were transplanted to small individual pots (15 cm^2 diam.) containing a mixture of 3 John Innes No 3 soil: 1 Viking: 1Perlite, and grown in a greenhouse. To obtain more floral buds per plant for the transformation, the first bolts were clipped.

2.2.3 Plant transformation

As described by Davis *et al.* (2009), *A. tumefaciens* strain GV3101 carrying *HvGI* pMDC32 construct was incubated in 10 ml of YEBs medium supplemented with 50 µg/ml kanamycin and 50µg/ml rifampicin. The tubes were placed on a 150 rpm shaker at 28°C for 24 h. Then, 10ml of bacterial culture was diluted in 500 ml of YEBs without antibiotics and placed back on the shaker for another 24 h. Then, 0.02% concentration of 100-200 µl Silwet L-77 was added to the *A. tumefaciens* culture and poured into the 500 ml beaker. Floral parts of *Arabidopsis* were dipped into *A. tumefaciens* inoculum for 10 seconds. Inoculated *Arabidopsis* plants were covered in separate plastic bags to retain the humidity. The inoculated plants were removed from their sealed bags the next day to avoid them rotting and dying. The plants were left in the greenhouse to grow until they dried out. Then, the seeds were harvested, keeping together the bolts from each pot.

2.2.4 Selection of transformed plants

Transformed plant seeds (T0) were surface sterilized and plated on an MS medium containing 1.5% agar and 30μg/ml selective agent Hygromycin. The plates were left in the dark at 4°C for 3 days and then moved into a plant growth room with 22°C and 16:8 h light:dark cycle for 10 days. Hygromycin-resistant seedlings were recognised by their having large green leaves and long roots in comparison to yellow leaf seedlings with a short root. Hygromycin -resistant seedlings were transferred to individual pots and grown to full maturity and produced the first transformed generation seeds (T1). Because *A. tumefaciens* normally targets female reproductive tissue only, T1 seeds should be heterozygous. According to the Mendelian segregation law, growing T1 seeds on MS Hygromycin medium should produced 3 Hygromycin resistant seedlings:1 Hygromycin sensitive seedling. Approximately 10 Hygromycin-resistant seedlings were transferred to soil to set T2 seed. T2 seed was grown again on an MS Hygromycin medium to screen for only 100% resistant plant lines, which were chosen to set T3 seeds. Homozygous T3 lines were used for circadian function analysis.

2.2.5. Leaf movement assays

To assay *Arabidopsis* leaf movement rhythms, surface sterilized seeds from each line were individually plated onto an MS agar medium and kept in the dark at 4°C for three days. Then the plates were moved into the growth room at 22°C and 12:12 h light:dark cycle. After 10 days, square agar blocks holding a single seedling were randomly cut and vertically arranged in three rows of 25 a compartmental square Petri dish plates (Barloworlds Scientific, UK). The plates were then moved back to the growth room and incubated at the same conditions for an additional 2-3 days. The seedlings were

transferred to the Sanyo MLR350 controlled growth chambers at dawn and imaged under constant light at 22°C for one week. The images were captured every 20 min using Sony Exwave HAD cameras (Sovereign International) programmed by Metamorph 6.0 software. The collected data produced by Metamorph were imported into the Biological Rhythms Analysis Software System (BRASS) and analysed as described by Gould *et al.* (2006).

2.2.6. Delayed fluorescence assays

Surface sterilized *Arabidopsis* seeds, from transformed lines and wild type, were sown onto MS agar in 96 well microtitre plates (Greiner Bio-one) and kept in the dark at 4°C for three days. The stratification plates were then transferred to the growth room at 22°C and 12:12 h light:dark cycle. After 16 days, the plates were placed in the imaging system at 22°C in constant 40μmol m^{-2} sec^{-2} RB light. Delayed fluorescence (DF) was measured as described by Gould *et al.* (2009) (see Chapter 6 for more details).

2.2.7. Flowering time measurements

Arabidopsis seeds were sown in plastic pots containing a standard mixture of 3 John Innes No 3: 1 Viking: 1 Perlite and stratified in the dark at 4°C for three days. Then, the seedlings were moved into a plant growth room and observed under 16:8 h light: dark and 22°C conditions. The flowering time was calculated as number of days until the plant revealed a 0.5 cm bolt. At this point, the total number of rosette leaves was counted. The collected data were analysed using Minitab 16th edition software and the means were compared using Tukey test at level $P \leq 0.05$.

2.3. Performance experiments

2.3.1 Growth conditions

Seeds of three barley genotypes (*Hordeum vulgare* l. cv. Golden Promise, Ppd-H1-wild type and ppd-H1 mutant) were grown in pots filled with a mixture of John Innes compost No.2 soil and 100 mg fertilizer (Osmocote®Exact®), saturated with water containing 0.2 g/l insecticide Intercept 70WS (The Scotts Company Ltd, UK). The plants were watered regularly. The pots were kept in a cold room (4°C) in the dark for three days before being transferred to controlled growth conditions to ensure the seeds germinated at the same time. The seedlings were grown for 15 days under different T cycles (light:dark cycle: 10:10 h (T20), 12:12h (T24), 15:15h (T30) and constant light) with 600 μmol m^{-2} s^{-1} light density at different temperatures 17, 22 and 27°C. The growth environment was controlled by using plant growth cabinets (MICROCLIMA, Climate chambers and cooled incubators, Sniders Scientific). The experimental pots were arranged in a randomised complete block design (RCBD) with four treatments (dark:light cycles), three different temperatures, three phenotypes, twenty replicates and all the experiments were repeated two times. The height of the plants was measured at 12 h time intervals.

2.3.2 Plant growth measurement

Ten randomly selected barley seedlings were collected at 7 and 15 days after germination and once the plants had split into roots, stems and leaves. Leaf area was scanned using Metamorph software, in order to measure the threshold area. Also, the plant materials were dried in paper bags at 80°C for 48 h to determine plant dry weight.

Several growth parameters were calculated according to Hunt (2002), using the classical plant growth analysis tool, which is available from www.aob.oupjournals.org. The mean relative growth rate (RGR, g g^{-1} d^{-1}) was determined as the rate of increase of the total dry matter per unit of plant dry mass. The leaf area ratio LAR (cm^2 g^{-1}), which is an index of the leafiness of the plant, was calculated as the ratio between the total leaf area and the total dry matter per plant; while the leaf weight ratio LWR (g g^{-1}), was estimated as the ratio between the total leaf dry weight and the total plant dry matter. The specific leaf area, SLA (cm^2 g^{-1}), was estimated as the mean leaf area exhibited per unit leaf weight, and the unit leaf rate, ULR (mg cm^{-2} day^{-1}), was calculated as the rate of increase of the total dry weight per unit of total leaf area. Directly, these four terms are identified and connected in the following equation:

$$RGR = ULR \times LAR = ULR \times LWR \times SLA$$

2.3.3 Chlorophyll content

Five randomly positioned plants were used for measuring chlorophyll content at 15 days after planting. At the time of plant harvesting, each leaf was weighed. The leaf samples were placed in 1.5ml eppendorf tubes. The leaf tissues were homogenised using a Qiagen tissue lyser (Adapter Set 2 x 96) and 700µl of 96% Ethanol was added during the homogenization. The tissue samples were centrifuged at 13000rpm for 2 minutes and the supernatant was transferred into a new test tube. The extraction was repeated with 300µl of 96% Ethanol, vortexed and after 2 min centrifugation at 13000rpm the supernatant was added to the first supernatant. A sub-sample of the supernatant was stirred well and promptly measured by Spectra Max 340 (Molecular Devices Corp., USA) and microplate reader (200µl), adjusted to 649 nm and 665 nm absorbance

wavelength. The blank sample 200µl 96% Ethanol was used as a calibration reading. Total chlorophyll was calculated according to the equations used by Ritchie (2006).

Chl a (µg/ml) = – 5.2007 x A_{649} + 13.5275 x A_{665} (± 0.03125 µg/ml)

Chl b (µg/ml) = 22.4327 x A_{649} – 7.0741 x A_{665} (± 0.02623 µg/ml)

Chl tot (µg/ml) = 17.232 x A_{649} + 6.4534 x A_{665}

The final concentration of the chlorophyll was expressed as µg/mg of fresh leaf tissue in accordance with Holden (1976) and Ritchie (2006).

2.3.4 Statistical analysis

Data were analysed using the general linear model (GLM) procedures in Minitab 16th edition software (Minitab Inc., State College, PA). The analysis was based on a randomised complete block design (RCBD). Where treatment were identified a significant source of variation, significant difference amongst pairs of means was examined using least significant difference (LSD) test at $P \leq 0.05$.

2.4. Delayed fluorescence experiments

2.4.1. Growth conditions

Seeds of three barley genotype (*Hordeum vulgare* l. cv. Golden Promise, Ppd-H1 wild type and ppd-H1 mutant) were grown in pots filled with a mixture of John Innes compost No.2 soil and 100mg fertilizer (Osmocote®Exact®), saturated with water containing 0.2g/l insecticide Intercept 70WS (The Scotts Company Ltd, UK). The plants were watered depending on their needs. The seedlings were grown in a 16:8 h light:dark cycle in a controlled plant growth room at 22°C and 80 mol m^{-2} sec^{-1} of light.

2.4.2. Measurement of DF

DF luminescence was monitored using the luciferase imaging system, as described previously by Gould *et al.* (2009). DF images were collected immediately preceding lights off using an ORCA-11-BT 1024 16-bit low light charged coupled device (CCD) camera cooled to -80°C (Hamamatsu Photonics; http://www.hamamatsu.com) and controlled by WASABI imaging software (Hamamatsu Photonics; http://www.hamamatsu.com). A 1 min time exposure was taken every 1 h. The images produced (RBF files) were converted to TIFF files using WASABI. DF images were quantified using the Metamorph package (Molecular Devices Ltd; http://www.moleculardevices.com) to measure integrated luminescence for specific regions within an image. Background intensities were calculated in each image from a region containing no plants and subtracted from each data point to give a final DF measurement.

2.4.3. DF rhythms' analysis

The barley plants were grown in 16:8h light:dark cycles at 22°C for three weeks. The leaves were cut into 1 cm pieces in a laminar air flow cabinet, placed in 25 compartmental squared Petri dish plates and floated on SDW containing 17.98 mg/ L Dithane protective fungicides. The plates were placed in the imaging system at 22°C in constant Red/ Blue light. DF images were collected every hour, as described above. The image acquisition and switching of the LED array was fully automated using the time-lapse function in WASABI software. The luminescence was normalized by subtracting the Y value of the best straight line from the raw Y value. The Excel data produced by Metamorph were imported into the Biological Rhythms Analysis Software System

(BRASS; available from http://www.amillar.org) and analysed using fast Fourier transformed non-linear least-square analysis (FFTNLLS; Plautz et al., 1997) on each DF time course series to generate period estimates and RAE.

CHAPTER 3: COMPROMISING THE BARLEY CLOCK BY KNOCKING OUT THE CLOCK COMPONENTS

3.1. Introduction

It is clear that *CCA1/LHY* and *TOC1* genes are essential components at the core of the *Arabidopsis* oscillator clock, while *GI* operates between the circadian oscillator and CO-FT pathway to control flowering by raising abundance of *CO* and *FT* mRNA. In *Arabidopsis thaliana*, the *cca1/lhy* double mutant flowers early and exhibits very short period rhythms that dampen rapidly in constant light conditions (Blazquez *et al.*, 2001). The loss of function mutations *cca1, lhy* and *toc1-2* also display short period and early flowering phenotypes under short day (SD) conditions (Strager *et al.*, 2000; Mizoguchi *et al.*, 2002; Niwa *et al.*, 2007). More importantly, both *toc1/cca1* and *toc1/lhy* double mutants displayed significant earlier flowering phenotypes compared with each single mutant. In addition, the *cca1/lhy/toc1* triple mutant exhibits practically the same degree of early flowering phenotype as the *cca1/lhy* double mutant and an arrhythmic phenotype under constant light conditions (Ding *et al.*, 2007; Niwa *et al.*, 2007). On the other hand, the *gi* loss of function allele causes a phenotype with an extremely late flowering and decrease in expression of *CCA1* and *LHY* under long day (LD) conditions (Searle and Coupland, 2004; Ausin *et al.*, 2005, Fujiwara *et al.*, 2005; Mizoguchi *et al.*, 2005) suggesting an alternative none circadian route by which GI alters flowering time.

The expression of the main clock genes (*CCA1/LHY, TOC1, GI*) in barley plants will be knocked down by using RNA-interference (RNAi) technique, which is an efficient method of silencing target genes using hairpin RNA (hpRNA) constructs containing sense and anti-sense arms (Wesley *et al.*, 2001; Helliwell and Waterhouse, 2003). The

aim is to generate clock mutants in barley and investigate their function on the barley clock and flowering time.

The constructs were cloned using Gateway cloning technology, which provides a fast and highly efficient method of transferring DNA sequences among multiple vector systems for functional analysis of genes and protein expression (Himmelbach *et al.*, 2007). The silencing constructs were transformed into the barley plants (Figure 3.1) (Wesley *et al.*, 2001) by cloning the genes into the pBract207 construct (Figure 2.2, A), which is specifically designed as a destination vector for this technology. This vector has a pGREEN backbone designed for plant transformation via *Agrobacterium*. The vectors contain two LR cloning sites that are separated by introns (http://www.bract.org). This design, following the LR reaction step, results in a hairpin RNA loop structure. The resulting dsRNA initiates the RNAi pathway, thus efficiently silencing the target gene (Figure 3.1). The RNAi pathway is a natural defence mechanism in plants against the dsRNA viruses. The dsRNA is cut into small fragments (20-25bp) by dicer enzymes. One of the two strands from each fragment is captured by an RNAi silencing complex (RISC). This allows the RISC to bind to a complementary region of the target mRNA. The catalytic components of the RISC cleaves the target mRNA. This causes degradation of the target mRNA. Thus, no protein is translated and the gene is efficiently silenced (Wesley *et al.*, 2001). These silencing constructs will be delivered into barley, a single gene at a time by using *A. tumefaciens* strain AGL1 as a vector. AGL1 is an aggressive strain of *A. tumefaciens* and it contains a binary vector pSoup (Figure 2.2, C), which provides replication functions for the pGreen binary. It also carries a gene that confers resistance to tetracycline in the *A. tumefaciens* and genes involved in the mobilisation of the T-DNA into the plant host. This transformation process occurs at the embryo stage and surviving plantlets will be grown on a Hygromycin-containing medium to select

positive transformants. Seeds will be produced from the positive transformed plants and homozygous lines will be selected in the T2 generation. Then experiments similar to those described by Dodd *et al.* (2005) will be conducted in order to measure the performance such as chlorophyll content, fresh and dry shoot weight, leaf area and levels of key metabolites, comparing *CCA1/LHY, TOC1* and *GI* mutant lines with wild type barley plants. We will also investigate circadian and flowering phenotypes with the aim of seeing if these genes have the same function in barley as those in *Arabidopsis*.

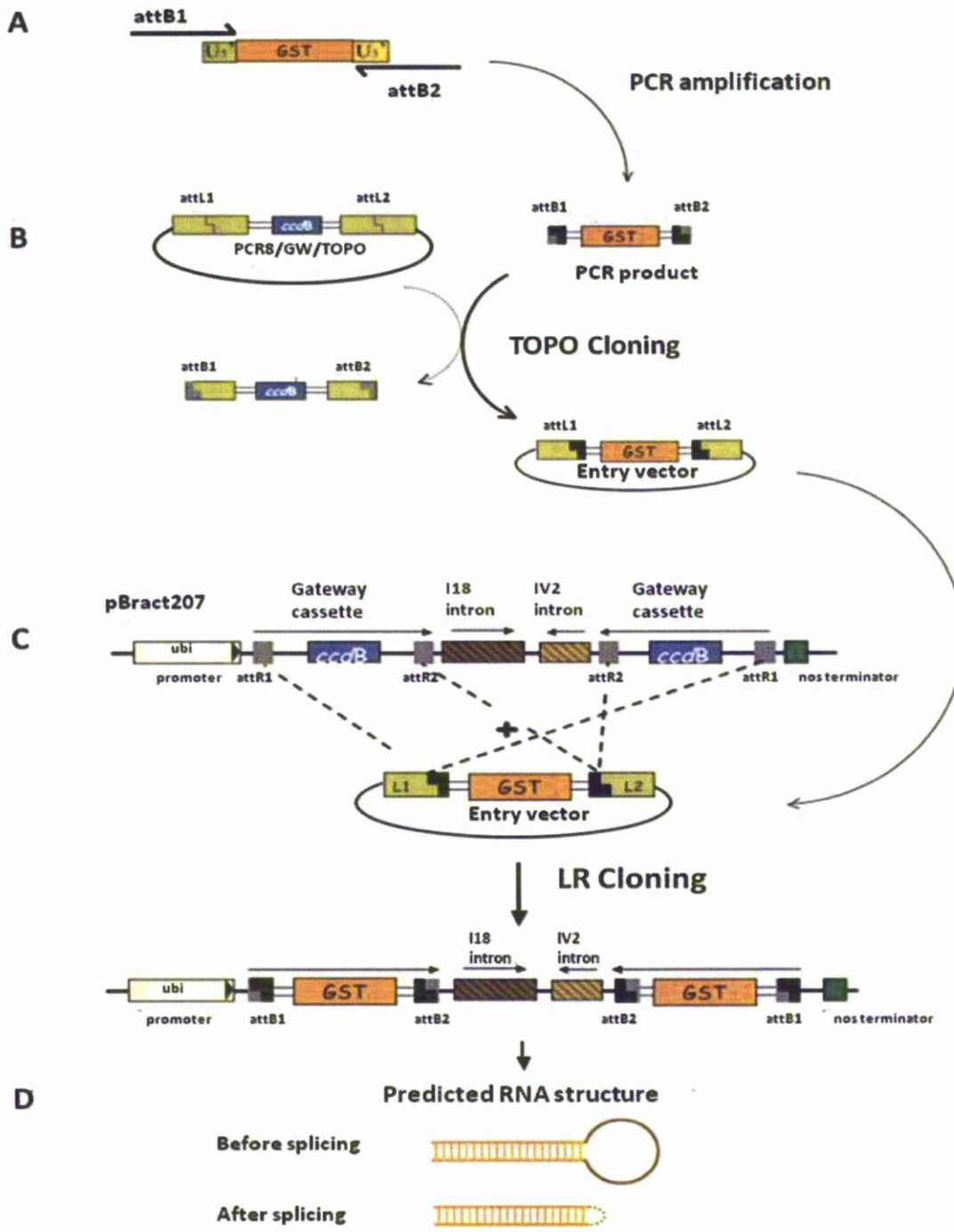


Figure 3.1. The hairpin RNA strategy. **(A)** Amplification for adding the recombination site attB. **(B)** Cloning of a GST as a Gateway™ entry clone by TOPO cloning. **(C)** Cloning GSTs into the hpRNA vector by LR cloning. **(D)** Predicted structure of the hairpin RNA when expressed in the plant cells.

3.2. Results

To investigate whether *TOC1* and *GI* genes have the same function in barley as those in *Arabidopsis*, the *TOC1* and *GI RNAi* silencing constructs were built using the pBract207 vector. The *TOC1* and *GI* fragments were amplified and cloned into the pBract207 vector using gateway LR recombination reaction. The *RNAi* constructs were transformed into the immature barley embryos via *Agrobacterium tumefaciens* (strain *AGL1)*. *Agrobacterium*-infected embryos were transferred to an induction medium with selective agents and incubated in the dark at 23-24°C for 6 weeks. Then the embryo-derived calluses were transferred to a transition medium to start production of shoots. The first embryos transformed did not produce green areas when they were transferred into the transition medium. A possible reason was that the embryos were too small, possibly due to infection of the donor plants by powdery mildew fungus. Therefore, the donor plants were sprayed with Dithane protective fungicide every week to prevent the development of any infection.

After that, transformation was repeated twice using a total of 625 immature embryos for each *RNAi* construct. Each time, a few embryos developed and produced a green area in the transition medium, but the green area turned yellow and died immediately when the embryos were transferred into regeneration media to generate more roots and shoots (Figure 3.2). The poor transformation could be linked either to poor quality of the donor plants or to the fact that they were sprayed with fungicide (Bartlett *et al.*, 2008).

To confirm whether the embryos had been transformed or not, DNA was isolated from three of the transformed embryos at the regeneration stage from each of the *TOC1*

and *GI* constructs. Then a PCR test was run with Hygromycin primers. The results of the PCR analysis subsequently showed that two embryos from each *TOC1* and *GI* construct contained the Hygromycin gene (Figure 3.3, A). To ensure that the transformed embryos had not been contaminated by other *bacterium* strains and to avoid poor transformation, *Agrobacterium* carrying the *RNAi* construct was re-examined by growing the *Agrobacterium* on the Eosin methylene blue agar medium and LXM medium. The *Agrobacterium* colonies turned green and yellow in the LXM medium and purple in the Eosin methylene blue agar medium; these results indicated that there was no contamination by other *bacterium* strains. The plasmids were extracted from a single *Agrobacterium* colony from each plate and re-tested by digestion with restriction enzymes, as described in the material and methods section (Figure 3.3, C). Also, a PCR test was run with *TOC1* (F-CCAGGTTAATTTCTCCGGTTCGACTGAC, R-ATACCGATGACCACACATTCTGCATTGA) and *GI* (F-TTCACTGAAGCGATGTAAGTGGGATGC, R-CTGAGAGCCTCGATAACCCCCATTTCT) specific primers to amplify the gene of interest. The obtained results show that each construct produced fragment of the gene of interest of the right size (~ 400 bp) (Figure 3.3, B).

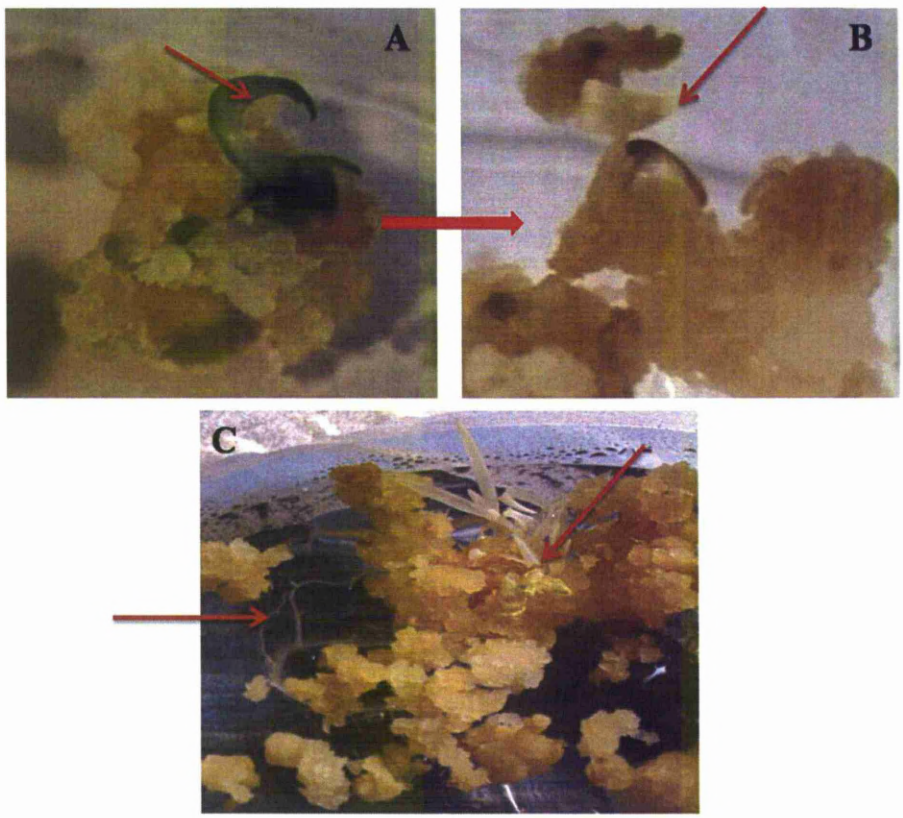


Figure 3.2. Immature barley embryos were transformed with the *TOC1 RNAi* construct in the regeneration medium. **(A)** Embryo-producing green shoot. **(B)** The green leaf that was produced turned yellow and died before producing a root. **(C)** The green leaf that was produced from a *GI RNAi* line turned yellow and died after producing a root.

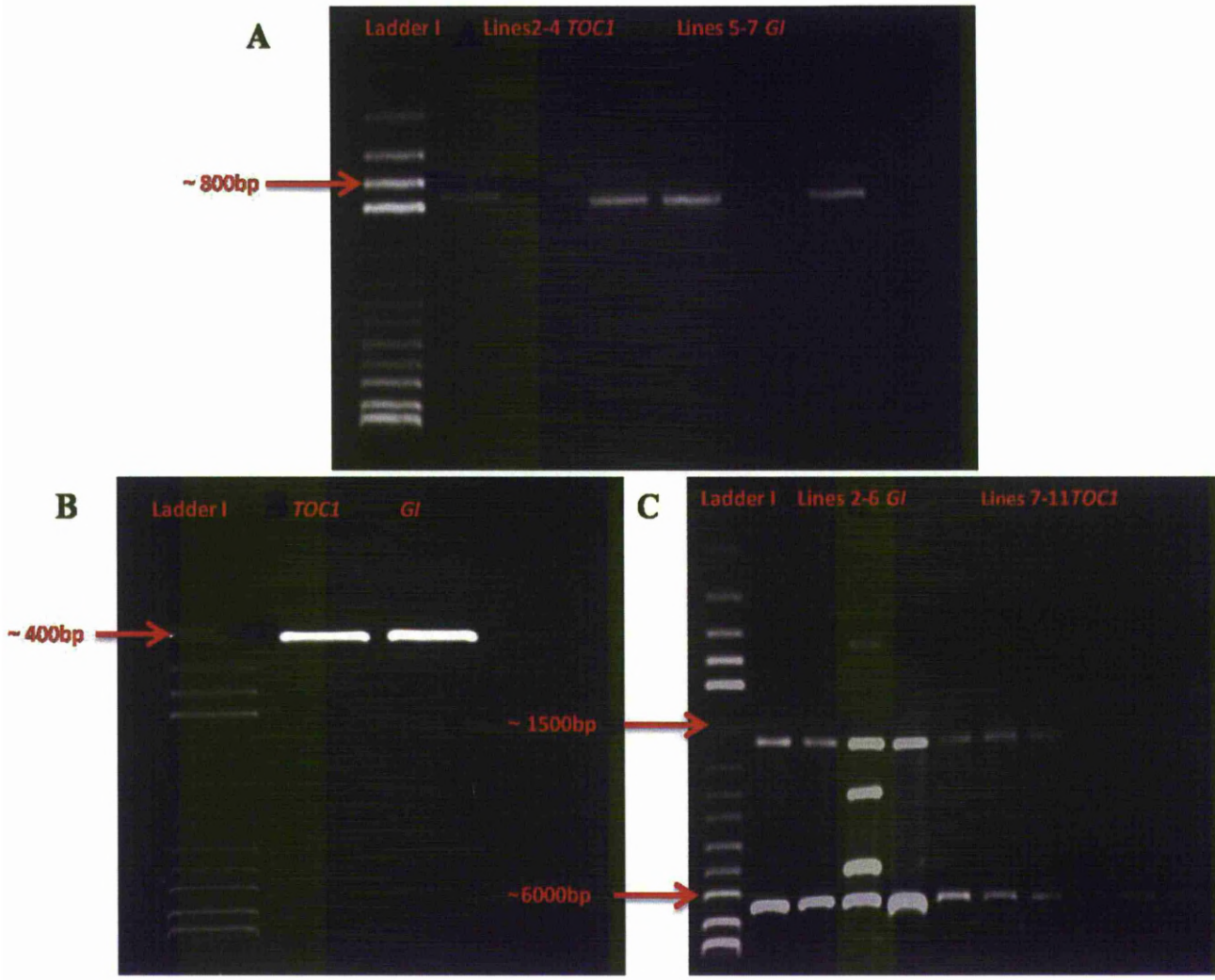


Figure 3.3. (A) 917bp PCR fragments of the *hpt* gene were amplified from embryos transformed by the *TOC1* and *GI RNAi* constructs using Hyg primers. Line 1 represents Hyper Ladder I, Lines 2-4 represent embryos with *TOC1-RNAi* constructs, Lines 5-7 represent embryos with *GI-RNAi* constructs, and Line 8 represents negative control. **(B)** 400bp fragments of the *TOC1* and *GI* genes were amplified using specific primers from the plasmid that has been extracted from *Agrobacterium*. **(C)** Digestion of *TOC1* and *GI RNAi* constructs with EcoRV restriction enzyme after extraction from *Agrobacterium*. Line 1 represents Hyper Ladder I, Lines 2-6 represent *GI-RNAi* constructs (6652+1627bp), and Lines 7-11 represent *TOC1-RNAi* constructs (6688+ 1627bp).

In order to examine whether the poor transformation was due to the target genes or the poor quality of the donor plant, the transformation was repeated at the John Innes Centre with high quality immature embryos. Some of the transformed embryos' plates were transferred to the University of Liverpool, while a few plates were left to continue development at the John Innes Centre. In these experiments, pBract202 was used as a negative control; this has the Hygromycin resistant gene driven by the *(CaMV)* 35s promoter. The reason for avoiding using pBract207 as a negative control was that pBract207 has the *ccdB* toxin gene, which allows negative selection of the destination vector. Bacteria carrying the *ccdB* gene will fail to grow. The *ccd*B gene has been replaced by target sequences in *RNAi* constructs by LR cloning. The transformation was quite successful (Figure 3.4). Nineteen independent transformed lines (25% transformation efficiency) were obtained from 75 embryos inoculated with the pBract202 which is an expected percentage; while 7 independent transformed lines (1.86% transformation efficiency) were selected from 375 inoculated embryos with the *TOC1 RNAi* construct; and 5 independent transformed lines (1.33% transformation efficiency) were developed from 375 embryos inoculated with the *GI RNAi* construct, which is far less than the expected.

Unfortunately, all of the obtained *GI* lines and most of the *TOC1* lines died immediately after transferral to the soil pots (Figure 3.5, A). The rest of the *TOC1* lines were extremely sensitive as a result of being transferred to soil pots and died later after a second transfer from small pots to large pots to grow and produce seeds while all control pBract202 lines reached full maturity and seed production (Figure 3.5, B). At the end of this experiment, none of the transformed lines with the RNAi constructs survived in the soil compared with the control ones in both places where the experiment was run

(University of Liverpool and John Innes Centre). At this stage, the reason behind the poor barley transformation cannot be identified. Silencing these genes in *Arabidopsis* plants does not have a negative effect such as killing the plant, and the pBract207 was previously used perfectly to silence other genes at the John Innes Centre. However, as *Ubi-1* is a strong constitutive promoter, it is possible that gene silencing has occurred and reduced levels of *TOC1* and *GI* expression interfered with barley regeneration and plant developments.

Figure 3.4. Immature barley embryos transformed with: **(A)** *TOC1 RNAi* construct. **(B)** *GI RNAi* construct. **(C)** *PBRACT 202* construct as control in the regeneration medium.

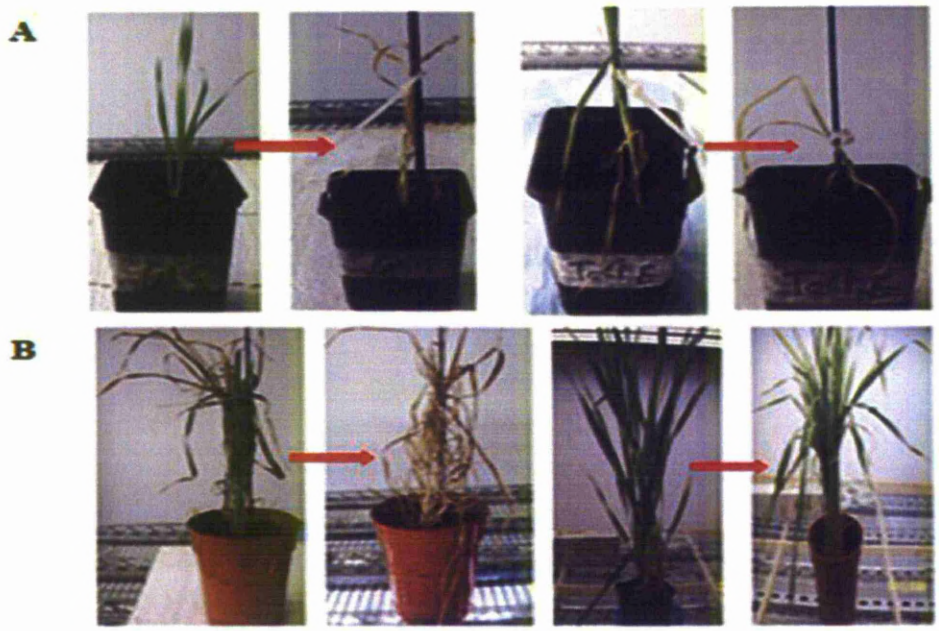


Figure 3.5. **(A)** *GI* and *TOC1 RNAi* lines died immediately after being transferred to the soil. **(B)** *TOC1 RNAi* lines died after being transferred into a large soil volumes and *pBract 202* lines grew well until maturity.

In order to evaluate the transformed lines, genomic DNA was extracted from leaf material by cutting 100 mg on the same day that the lines were transferred to the soil. Standard PCR analysis was carried out with the appropriate primers including Hygromycin (Hyg F-ACTCACCGCGACGTCTGTC, R-GCGCGTCTGCTGCTCCAT), *pBract207* introns sequence (*pBract207* IS F-TGGCGATTATTGCTCAGTTG, R-GGTAGAAGCAGAACTTACGTACA) and *TOC1* and *GI* specific primers. The results indicated that most of the lines transformed with *TOC1* and *GI* constructs showed the presence of the Hyg fragments 917bp (Figure 3.6A), the fragments of the genes of interest (400bp) (Figure 3.6B), and pBract 207 intron sequence fragments 123bp (Figure 3.7A). Although the plasmids were fully checked before transforming them into immature embryos, additionally, a PCR assay was run with *ccdB* primers (*ccdB* F-

ATGCAGTTTAAGGTTTACACC, R- ATGCAGTTTAAGGTTTACACC. The *ccdB* is a toxin gene used as a negative selection marker in pBract207, thus plants containing this gene might fail to grow. The *ccd*B gene should be replaced by target sequences in both LR cloning sites in the *RNAi* constructs by LR cloning. However, the target sequences sometimes flanked in only one LR cloning site. Thus, absence of the 307bp fragment of *ccdB* gene in transformed lines should confirm that the target sequences flanked in both LR cloning sites. The visualisation of the gel demonstrated that all of the *TOC1* and *GI* lines did not display the 307bp fragment of *ccdB* gene compared with control pBract207 (Figure 3.7B).

The silencing construct for the morning expression gene, *CCA1*, was built, tested with restriction enzymes, and fully sequenced but, unfortunately, there was not enough time to transfer the *CCA1 RNAi* construct into the barley embryos and examine if it had same side effects as the evening expression genes (*TOC1* and *GI*).

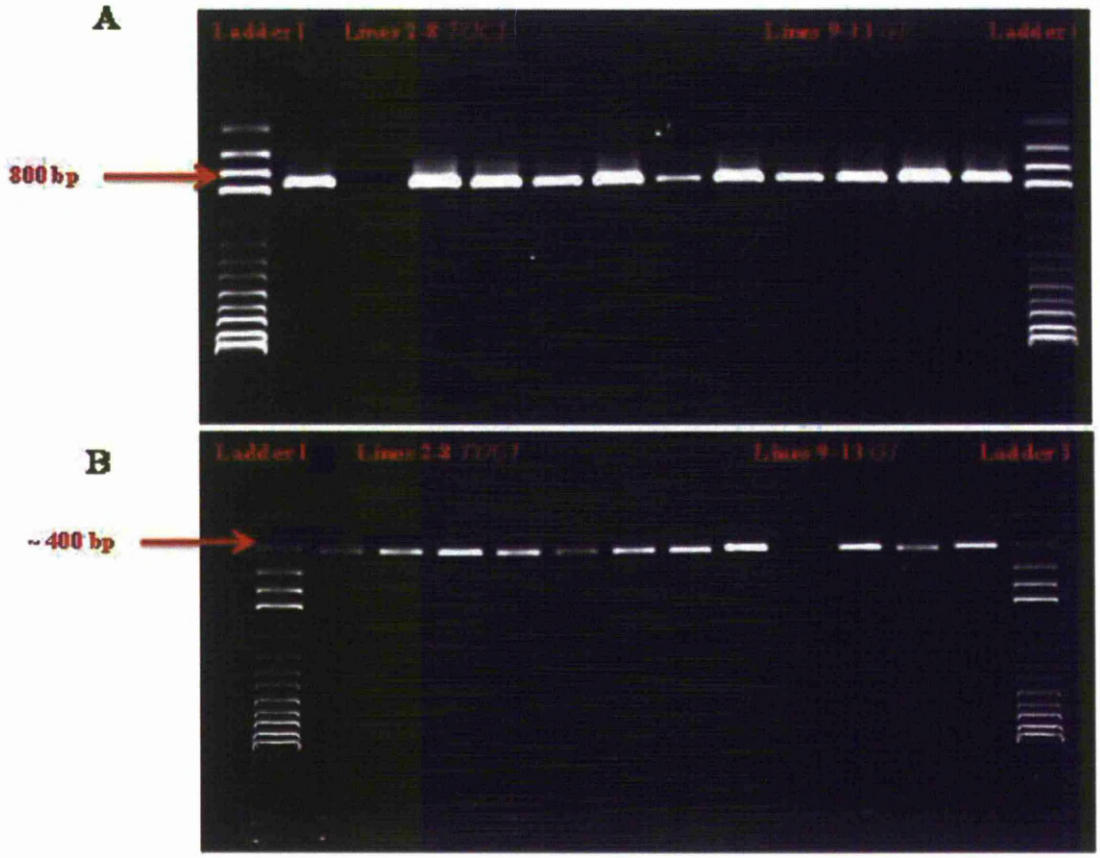


Figure 3.6. (A) 917bp PCR fragments of the *hpt* gene were amplified from genomic DNA which was extracted from *TOC1* and *GI RNAi* lines using Hyg primers. **(B)** 417bp fragments of the T-DNA sequence of *TOC1* and 403bp of the *GI* genes were amplified using specific primers from the transformed lines. Line 1 represents Hyper Ladder I, Lines 2-8 represent *TOC1-RNAi* lines, Lines 9-13 represent *GI-RNAi* lines, and Line 14 represents Hyper Ladder I.

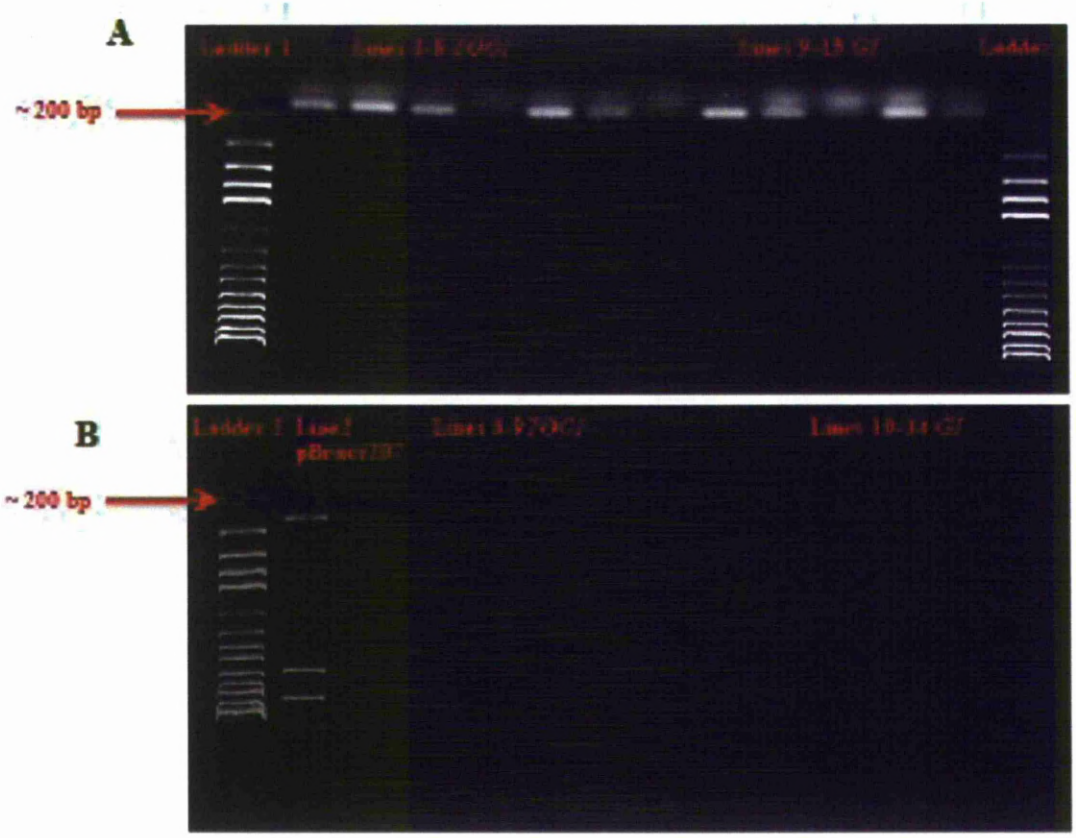


Figure 3.7. (A) 123bp PCR fragments of the *pBract207* intron sequence were amplified from genomic DNA which was extracted from *TOC1* and *GI RNAi* lines using *pBract207* intron sequence primers. Line 1 represents Hyper Ladder I, Lines 2-8 represent *TOC1-RNAi* lines, Lines 9-13 represent *GI-RNAi* lines, and Line 14 represents Hyper Ladder I. **(B)** 307bp fragments of the *ccdB* genes were amplified from genomic DNA extracted from the transformed lines using *ccdB* primers. Line 1 represents Hyper Ladder I, Line 2 represents pBract207 construct, Lines 3-9 represent *TOC1-RNAi* lines, and Lines 10-14 represent *GI-RNAi* lines.

3.3. Discussion

Barley genes *HvTOC1* and *HvGI*, homologous to *AtTOC1* and *AtGI* have been identified which are circadian regulated with peak expression in the subjective evening (Cotter, 2010). The roles that these genes play in the barley circadian clock have never previously been examined. In these experiments, the functions of both *HvTOC1* and *HvGI* genes in the barley circadian oscillator were investigated by knocking down the expression of these genes using the *RNAi* techniques. The most interesting finding is that silencing the evening genes in barley appears to have a negative effect on development and survival of the barley plants. The *HvTOC1* and *HvGI* RNAi transformed lines died early in the regeneration stage (Figure 3.2) or immediately after being transferred to the soil (Figure 3.5) compared to the control transgenic plants transformed with *pBract 202*. In contrast, *TOC1* loss of function in *Arabidopsis* plants displayed a short period and early flowering phenotype under short day (SD) whereas the *GI* loss of function displayed an extremely late flowering phenotype and decreased the expression of *CCA1* and *LHY* under long day (LD) conditions (Mizoguchi *et al.*, 2002; Mizoguchi *et al.*, 2005). This result could be interpreted as the *HvTOC1* and *HvGI* genes playing a role in the successful early development and establishment of the plant.

According to the preliminary results of PCR analysis using Hyg primers, two of three transformed embryos contained Hyg fragments (917bp) (Figure 3.3, A). Furthermore, PCR analysis of the *HvTOC1* and *HvGI* transformed lines with appropriate primers including Hygromycin, *pBract207* intron sequence, *ccdB* primers and *TOC1* and *GI* specific primers confirmed that most of the *HvTOC1* and *HvGI RNAi* lines were successfully transformed (Figures 3.6 and 3.7). However, the tissues were not tested for *Agrobacterium* contamination at this stage because it was assumed that there was no

Agrobacterium still present in the embryo and leaf tissues at the final transformation stages. These observations indicated that losing the full functionality of *HvTOC1* and *HvGI* genes may provide major disadvantage in the transformed plant. At the same time, there is little understanding of the behaviour of these genes in the barley circadian oscillator and input and output pathways. In other words, the effects of knockdown of evening genes in barley plants are difficult to interpret as a result of the difference between the data obtained here and that from other plant species. One future approach may be to use an inducible RNAi construct that allows silencing later in the plant's development, once it has established itself, or in subsequent generations. It would also be interesting to see if morning components of the clock, ie. CCA1 had the same effect.

CHAPTER 4: OVER-EXPRESSION OF BARLEY *GIGANTEA* IN *ARABIDOPSIS* PLANTS

4.1. Introduction

GIGANTEA (*GI*) is another clock-associated gene that plays a pivotal role in circadian oscillations and floral pathways to promote flowering-time by rising abundance of *CO* and *FT* mRNA. It is a gene encoding a novel nuclear-localised protein (1,173 amino acids) which has no homology with any proteins of known biochemical function in the databases (Mizoguchi *et al.*, 2005). Mutations in the *Arabidopsis GI* gene (*AtGI*) cause a pleiotropic phenotype with effects on flowering in response to photoperiod, phytochrome B signalling, the circadian clock, and carbohydrate metabolism (Song *et al.*, 2007). Both a *gi* loss-of-function and a *GI* over-expression mutant displayed short-period rhythms with lower amplitudes and altered flowering time (Mizoguchi *et al.*, 2002; Mizoguchi *et al.*, 2005). The *GI-ox* displayed an early flowering phenotype under both LD and SD and had increased expression of both floral integrator genes *FT* and *SOC*, whereas loss of *gi* function resulted in a late flowering phenotype under LDs and phenotypes with lowered in levels of *LHY, CCA1, CO* and *FT-RNA*, that rapidly dampen in LL (Fowler *et al.*, 1999; Mizoguchi*et al.*, 2002; Mizoguchi *et al.*, 2005). Recent studies have revealed that *GI* can regulate *FT* expression independently of *CO* through regulated miR172 or by binding to three *FT* repressors: *SVP, TEM1* and *TEM2. GI* is also capable of directly activating *FT* expression by binding onto its promoter region, which is near the *SVP* binding sites (Jung *et al.*, 2007; Sawa and Kay, 2011).

GI was also proposed to play a critical role in temperature compensation in the *Arabidopsis* clock, by extending the temperature range over which robust and accurate

rhythmicity can be maintained (Gould *et al.*, 2006). *AtGI* was also involved with SPINDLY as a negative regulator of gibberellin signalling (Tseng et al., 2004; Dunfort *et al.*, 2005). Recently, Dalchau *et al.* (2011) reported that *GI* operates as part of a sucrose-signalling network and allows metabolic input into circadian timing in *Arabidopsis*. *GI* is also involved in regulation of the wall ingrowth development downstream of the stress signalling pathways in *Arabidopsis* transfer cells. The amount of phloem parenchyma containing wall ingrowths was reduced 15-fold in gi-2 and gi-3 mutants compared with wild type. Discrete papillate wall ingrowths were formed and failed to develop into branched networks in gi-2 plants. Wall ingrowth development in gi-2 was not recovered by exposing these plants to high light or cold conditions. On the contrary, over-expression of *GI* in the gi-2 background restored the wall ingrowth deposition to wild type levels (Edwards *et al.*, 2010).

GI is highly conserved in seed plants, including monocotyledonous plants such as rice (*Oryza sativa*) and barley (*Hordeum vulgare* L.). Genetic mapping showed that *GI* is found as a single copy gene in barley (3HS) and had 94% and 79% similarity and 88% and 66% identity with *OsGI* and *AtGI*, respectively (Dunfort *et al.*, 2005). Additionally, *HvGI* is unconnected to the other identified photoperiod genes of barley (ppd-h1 2hs and ppd-h2 0n 1hl) (Dunfort *et al.*, 2005). Moreover, the expression patterns of the barley GI genes (HvGI) are likely to be regulated by the circadian clock in a similar pattern to *AtGI* under constant light conditions (Cotter, 2010). Serikawa *et al* (2008) reported that rhythms of reporter genes *AtCCA1:luc* and *AtTOC1:luc* were dampened in over-expressing *LgGI* plants, while the rhythms of both reporters were completely abolished in the loss of function of the *LgGI* plant. Thus, unlike *AtGI*, *LgGI* is essential for the *Lemna* circadian oscillation. Furthermore, over-expression of the *BdGI* gene in the *Arabidopsis*

gi-2 mutation fully rescued the late flowering phenotype of this mutation, demonstrating that *BdGI* also plays a role in promoting flowering (Hong *et al.*, 2010).

As a first step towards examining if barley *GI* performs the same function as *Arabidopsis GI*, the barley gene was cloned into an over-expression *PMDC32* construct and transformed into *Arabidopsis* WS (wild type) plants and *gi-11* mutants in the WS ecotype. The *gi-11* mutant was a single T-DNA insertion mutant generated by using the pGKB5 tagging vector and isolated in a screening method described by Fowler *et al.* (1999). The rescue of the *Atgi-11* mutant line by the *HvGI* gene will help to determine the biological function of *HvGI* gene and if this is conserved in cereal crops. If *HvGI* is involved in the same output pathways as *AtGI* then the expected result would be short-period rhythms with lower amplitudes and early flowering phenotypes under both LD and SD.

4.2. Results

The over-expression *PMDC32* constructs (Figure2.3 B) carrying the 2X35S:*HvGI* gene were transferred into *Arabidopsis* WS and *gi-11* mutant lines via the floral dip method. All experiments planned to compare *HvGI* lines with wild type WS and a *gi-11-ox* line, which was established by crossing the *gi-11* mutant with transgenic plants of the *35S:GI* (Oliverio *et al.*, 2007). The mutant phenotype of *gi-11* was fully rescued in the *gi-11-ox* line. If the *HvGI* gene also has the ability to rescue the *gi-11* mutant phenotype, the transgenic lines should have a similar phenotype to the *gi-11-ox* line, thus *gi-11-ox* was used as a control instead of *gi-11* mutant. For the barley *HvGI* lines, out of the 53 primary transformants, 22 were identified as homozygous lines carrying an insert at a single locus. Only 2 homozygous lines (11.11% transformation efficiency) were obtained from 18 of

the *HvGI* transgenic lines in the *gi-11* mutant, and 20 homozygous lines (57.14% transformation efficiency) were obtained from 35 of the *HvGI* transgenic lines in the WS ecotype. Only 5 transgenic lines were used for further analysis because of limited camera space. Leaf movements and delayed fluorescence experiments were carried out to analyse the circadian phenotype of these lines. Furthermore, flowering times in these lines were measured. All experiments were independently repeated at least twice.

4.2.1 Leaf movement assays

To investigate whether the barley orthologue of *GI* has an analogous function to *AtGI*, circadian rhythms of the *HvGI* lines were first assayed by using leaf movement technique. The transgenic lines and the control plants were entrained under 12:12 h light:dark at 22°C for 10 days. The free-running periods of leaf movement rhythms were measured at 22°C in constant light conditions. The data were displayed by plotting the individual period estimates for leaf movement rhythms against their relative amplitude errors (RAE) - the variability of period estimates as well as the robustness of the rhythm. RAE is a measure of rhythm robustness, which can vary from 0 (robust rhythm) to 1 (no rhythm), with values above 0.5 meaning that there is no statistically significant rhythm (Dowson-Day and Millar, 1999). A wide scattering of data points on the graph indicates a loss in the precision of the clock, while tightly clustered data points with low RAE are associated with robust rhythms and a precise clock (Gould *et al.*, 2006). It was noted that the data points of *HvGI* transgenic lines in WS and the *gi-11* mutant formed a tight cluster as the control plant and their period almost completely matched the wild type WS and *gi 11–ox* (Figure 4.1) (Table 4.1). It was found that *35S:HvGI* 2-4 and 7-3 lines were capable of rescue the *gi-11* mutation (Figure 4.1, A). This result supports the idea of

conservation of the *GI* gene among monocot and dicot plants, and *HvGI* might play a role in the circadian clock similar to that of *AtGI*.

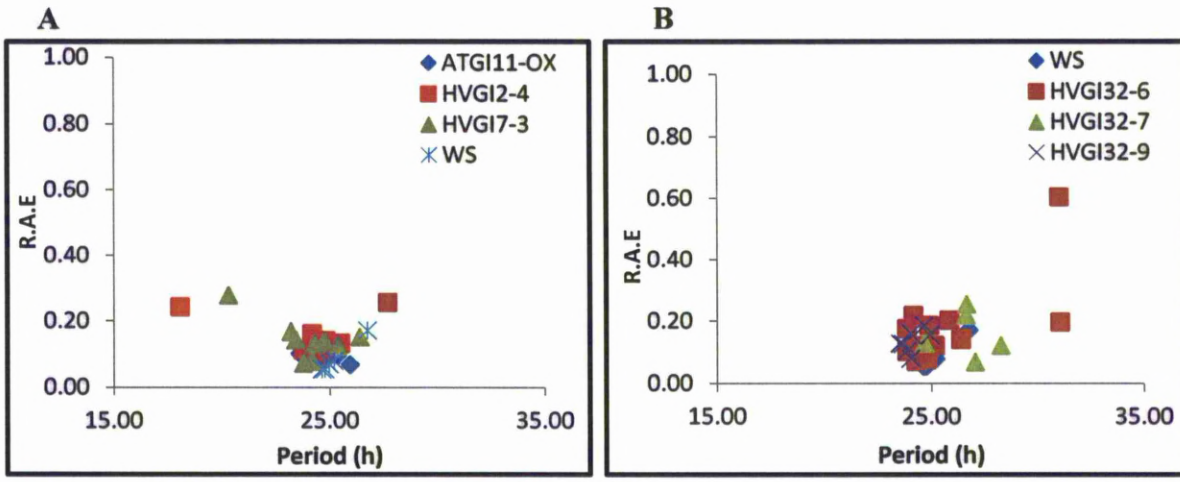


Figure 4.1. Analysis of circadian phenotypes of *Arabidopsis* transgenic lines. **(A)** Leaf movement of *HvGI* transgenic lines in *AtGI-11*(n=30). **(B)** Leaf movement of *HvGI* transgenic lines in WS (n=30). All plants were grown on MS agar at 22°C under 12:12 light:dark cycle for 10 days before the transfer to continuous light at 22°C, where leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its RAE.

4.2.2 DF analysis

Delayed fluorescence supplementary experiments were conducted to support the leaf movement results. Transgenic lines and control plants were entrained at 12:12 h light:dark cycles at 22°C before being transferred to continuous light conditions for circadian rhythm measurement. Then, the plates were incubated in an imaging camera system at 17°C under 40 µmol m^{-2} sec^{-1} constant RB light for 5 days with 1min exposures were set every one hour after switching LED light off. The images were processed using Metamorph 6.0 image-analysis software (Molecular Devices). The data produced by Metamorph were normalised by BRASS. The DF results display that almost

all of transgenic lines exhibit robust DF rhythms that peak earlier than wild type WS and *AtGI-11-ox* (Figure 4.2A and C). However, the *35S:HvGI* transgenic lines in WS had a period longer by 1 hr and 1.30 hours than wild type WS (Table 4.1, Figure 4.2D). Whereas, the *35S:HvGI* transgenic lines in *AtGI-11* exhibit robust DF rhythms that are weaker than wild type WS and *AtGI-11-ox* (Figure 4.2A) with a period shorter by 2 and 2.30 hours than wild type WS and *AtGI-11-ox* (Table 4.1, Figure 4.2B). Both leaf movement and delayed fluorescence data demonstrated that *HvGI* rescues the circadian arrhythmic of null allele gi-11 mutation. These results support the theory that *HvGI* might have a function in the barley circadian clock similar to that of *AtGI.*

Table 4.1. Estimation of the circadian period for leaf movement and delayed fluorescence.

	LM Data				DF Data			
Line	**Period (h)**	**RAE**	**n**	**Rh%**	**Period (h)**	**RAE**	**n**	**Rh%**
AtGI-11-ox	24.56	0.10	30	93.33	24.72	0.26	24	100
HvGI-2-4	24.14	0.12	30	80	22.13	0.38	24	83.33
HvGI-7-3	23.42	0.10	30	63.33	23.07	0.31	24	70.38
WS	24.25	0.05	30	86.67	23.82	0.28	24	91.67
HvGI32-6	24.26	0.11	30	83	24.66	0.19	24	100
HvGI32-7	26.90	0.14	30	66.67	25.13	0.23	24	95.83
HvGI32-9	24.00	0.11	30	70	24.59	0.26	24	100

Period estimated, relative amplitude errors (RAE) and percentage of rhythmicity were calculated from 30 leaf traces per *Arabidopsis* line for leaf movement, and 24 group seedlings for DF.

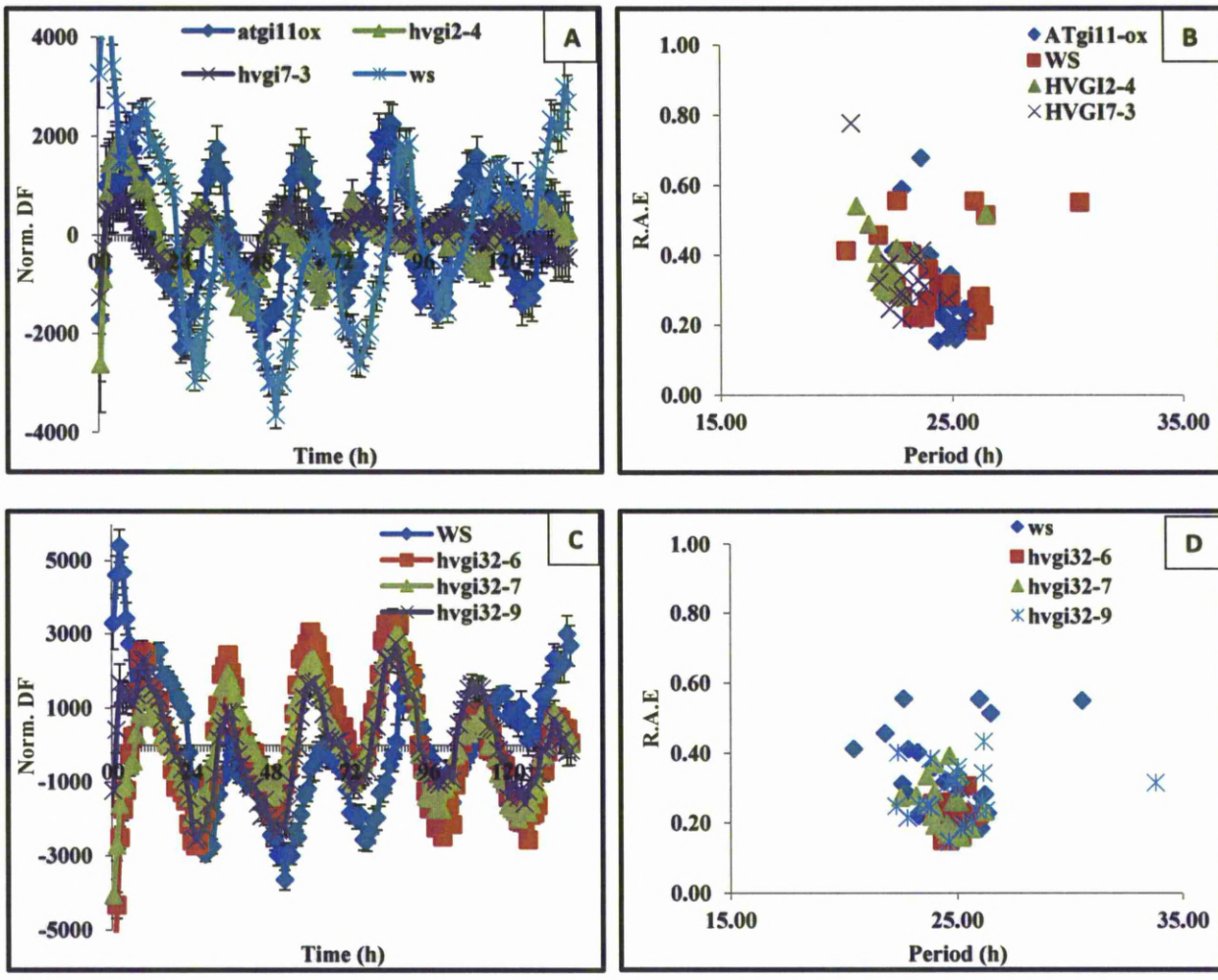


Figure 4.2. Delayed fluorescence rhythms for *HvGI* transgenic lines. **(A)** Normalized averages of DF rhythms for *HvGI* transgenic lines in *AtGI-11*. **(B)** Circadian period estimated for DF plotted against RAE. **(C)** Normalized averages of DF rhythms for *HvGI* transgenic lines in WS. **(D)** Circadian period estimated for DF plotted against RAE. All *Arabidopsis* seedlings were grown on MS media and entrained under 12:12 light: dark cycle for 16 days before transferral to constant RB light (40μmol m^{-2} sec^{-1}) for imaging. The average of DF was calculated from 24 groups of seedlings every 1 h for 96 h.

4.2.3 *HvGI* altered flowering time in *Arabidopsis*

Flowering time was measured by counting the days from seed germination to flowering. The transformed lines flowered significantly earlier in the presence of *HvGI* than wild type. The advance ranged from 3 days in the *35S:HvGI7-3* line to 10 days in the *35S:HvGI2-4* line in comparison with *AtGI-11-ox* control plants (Figure 4.3); whilst the advance ranged between 5 days in *35S:HvGI32-9 line* to 10 days in *35S:HvGI32-6* in comparison with WS control plants (Figure 4.3). The regulation of vegetative development of transgenic plants was also affected by the constitutive expression of the *HvGI* in *Arabidopsis.* The date from graphing leaf counts over time also show that *35S:HvGI* transgenic lines in WS flowered at rosette leaf number of 7.5, and the control plants WS flowered at rosette leaf number 11.62 under LDs (Figure 4.4); whereas, the *35S:HvGI2-4* line flowered at rosette leaf number 6.63 and the *35S:HvGI7-3* line flowered at rosette number 11.75 compared with the *gi-11* mutant plants and *AtGI-11-ox*, which flowered at rosette leaf number 25.38 and 13.5, respectively, under LDs (Figure 4.4). The late flowering phenotype in *Arabidopsis* that resulted from the late flowering mutant *gi-11* was completely rescued by over-expression of the *HvGI* gene. These results clearly demonstrate that the role of the *GI* gene in flowering induction is conserved in *Arabidopsis* and barley.

Overall, the obtained results support the theory that *HvGI* gene is functionally similar to the *Arabidopsis AtGI* gene orthologue, functioning as a floral promoter, and likely plays a central role in the barley central oscillator. Also, the *HvGI* protein shares an amino acid identity of 74% with *AtGI*, which might allow interaction with the *Arabidopsis* flowering pathways and clock machinery.

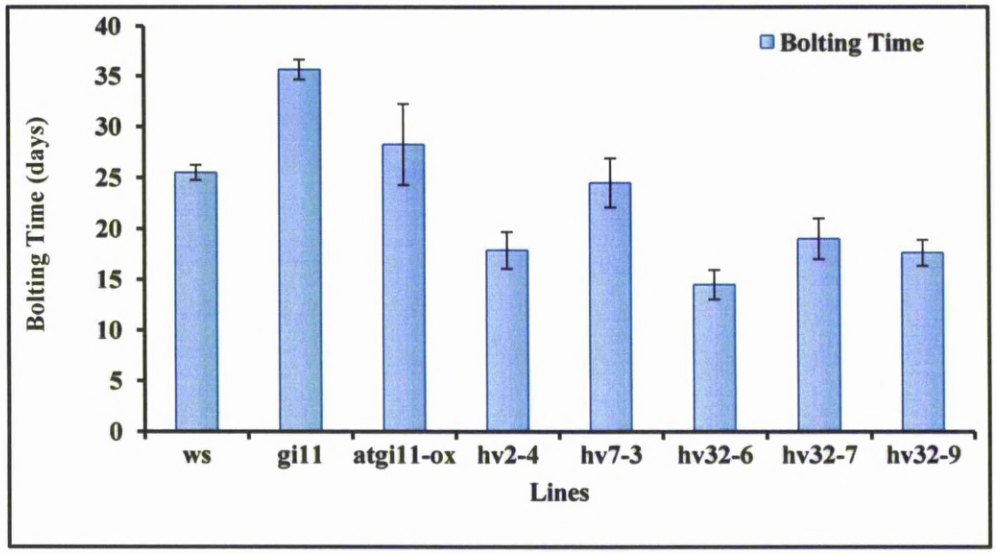


Figure 4.3. Bolting time for the *35S:HvGI* transgenic lines in *Atgi-11* mutant plants and *35S:HvGI* transgenic lines in WS plants. Mean (±SE) of bolting time for control (WS, Atgi-11 and Atgi-11-ox) and *35S:HvGI* transgenic lines. Plants were grown at 22°C under 16:8 h light:dark conditions.

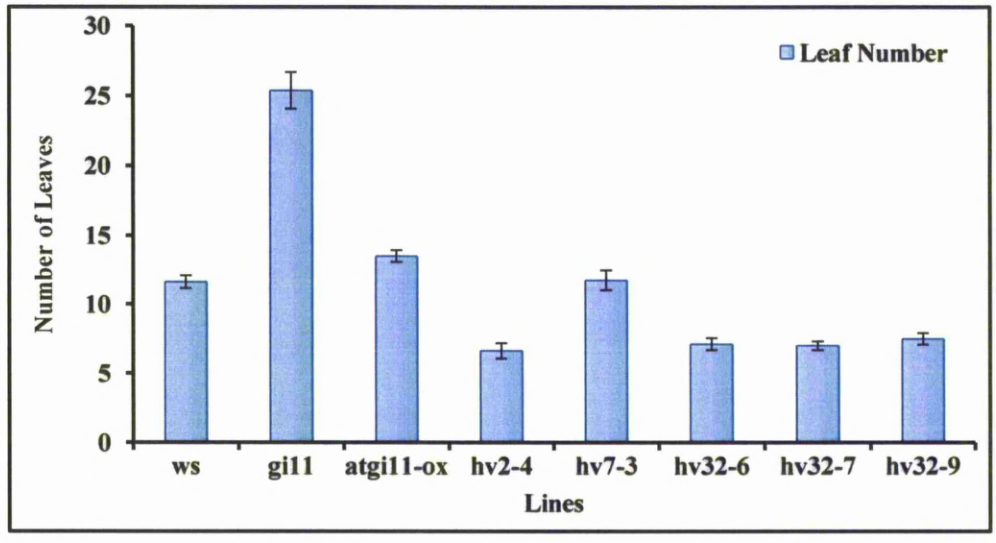


Figure 4.4. Number of rosette leaves for the *35S:HvGI* transgenic lines in *Atgi-11* mutant plants and *35S:HvGI* transgenic lines in WS plants. Mean number (±SE) of rosette leaves for control (WS, Atgi-11 and *Atgi-11-ox*) and *35S:HvGI* transgenic lines. Plants were grown at 22°C under 16:8 h light:dark conditions.

4.3. Discussion

There is pronounced evidence that *AtGI* acts in blue light signalling and has biochemically separable roles in the circadian clock and flowering time regulation (Martin-Tryon *et al.*, 2007). It is also involved in starch accumulation, hypocotyl growth under continuous red light, and resistance to stress (Eimert *et al.*, 1995, Paltiel *et al.*, 2006, Oliverio *et al.*, 2007). In this chapter, a range of clock analysis protocols were applied to define the circadian functions of the *HvGI* gene by investigating the effect of *35S:HvGI* in *Arabidopsis* plants and also exploring its role in promoting the time of flowering by using *Arabidopsis* late flowering *gi-11* mutant. Constitutive expression of *HvGI* in *Arabidopsis* results in rescue of the circadian arrhythmic and late flowering phenotype of null allele *gi-11* mutation, suggesting that the function of the *HvGI* protein has been conserved through evolution dicots to the monocots species in the higher plant kingdom.

In *Arabidopsis*, the *AtGI* gene expression is under the control of the circadian clock with a peak expression in the evening and photoperiods (Fowler *et al.*, 1999). The expression patterns of *OsGI, HvGI, BdGI* and *AtGI* genes are regulated by the circadian clock in a similar pattern under LDs and SDs (Hayama *et al.* 2003, Dunfort *et al.*, 2005, Cotter, 2010, Hong *et al.*, 2010). The results of leaf movement analysis show that *35S:HvGI* transgenic lines in the *Arabidopsis* plants have leaf movement periods almost completely matched to the wild type WS and *Atgi-11–ox* plants (Table 4.1). However, the observations are different in DF experiments, where almost all of the transgenic lines exhibit robust DF rhythms that peak earlier than wild type WS and *Atgi-11-ox* (Figure 4.2 A and C). The *35S:HvGI* transgenic lines in WS had a period longer by 1 hr and 1.30 hours than the wild type WS (Table 4.1, Figure 4.2, D); whereas the *35S:HvGI* transgenic

lines in *AtGI-11* exhibited robust DF rhythms that had a period shorter by 2 and 2.30 hours compared with wild type WS and *AtGI-11-ox* (Figure 4.2, A). The possible explanation for this might be the longer period of the *35S:HvGI* transgenic lines in WS resulting from the interaction between both *AtGI* and *HvGI* in *Arabidopsis* clock machinery; while the shorter period of the *35S:HvGI* transgenic lines in *gi-11* background might be due to *HvGI* restoring the *Arabidopsis* clock machinery. The*35S:HvGI* transgenic lines in *gi-11* background exhibit robust DF rhythms that are weaker than wild type WS and *AtGI-11-ox*, which is similar to the barley DF signal, suggesting that *HvGI* could interact and function within the machinery of the *Arabidopsis* clock in a similar way to the *AtGI*.

Over-expression of the *GI* gene in the *Arabidopsis* plant exhibited an early flowering phenotype under both LD and SD (Fowler *et al.*, 1999); whereas over-expression of *OsGI* in the transgenic rice gene increased Hd1 expression and reduced Hd3a expression, which led to a late flowering phenotype under both SD and LD (Hayama *et al.*, 2003). In this study, over-expression of the *HvGI* gene in the *Arabidopsis* late flowering mutant *gi-11* background completely rescued the late flowering phenotype; and *35S:HvGI* transgenic lines in WS flowered earlier than wild type under LDs, indicating that the *HvGI* gene is also involved in regulation of the photoperiodic pathway. This outcome may be explained by the fact that the *HvGI* protein shares an amino acid identity of 74% with *AtGI*, which might allow interaction between the *Arabidopsis* flowering pathways. These finding are consistent with those of previous research by Hong *et al.* (2010), which found that over-expression of the *BdGI* gene in the *Arabidopsis gi-2* mutation was fully rescued the late flowering phenotype of this mutation, demonstrating that *BdGI* also plays a role in promoting flowering time. The *BdGI* protein was also able to interact with other proteins

such *as COP1, FKF1, SPY*, and *ZTL*. It seems that these obtained results are due to the *HvGI* gene, which had a 91% sequence identity with the *BdGI*, which permitted its protein to be involved in *Arabidopsis* flowering pathway in similar ways.

This study demonstrates that the *HvGI* gene is most likely the *Arabidopsis AtGI* gene orthologue, functioning as regulator in both circadian clock oscillation and photoperiodic pathways. This suggests that expression of the *HvGI* gene can be manipulated by plant breeders as targeted genetic modification of flowering time traits to enhance the crop yield.

CHAPTER 5: QUANTIFICATION OF THE IMPORTANCE OF LIGHT AND TEMPERATURE COMPENSATION OF THE CIRCADIAN CLOCK IN THE ENHANCEMENT OF THE GROWTH AND FITNESS OF BARLEY PLANTS

5.1. Introduction

Circadian clocks are capable of being entrained by environmental stimuli such as light and temperature, which are frequently referred to as "zeitgebers" or "time-givers". These exogenous cues can reset the clock mechanism and thus adjust the output rhythms to changes in daylight hours as the seasons of the year progress (Jones, 2009). The clock mechanism can respond differentially to zeitgebers at definite times of the day. For instance, applying light pulses during subjective day would not cause any phase shift of the clock. However, applying pulses of light during the early morning could cause phase advances, although applying pulses of light after subjective dusk displays a phase delay (Devlin and Kay 2001). Resetting of the oscillator by light signals at an appropriate time of day is known as "gating". This hypothesis suggests that light input is gated by the oscillator; hence the oscillator regulates its own resetting (Devlin, 2002).

In *Arabidopsis*, *CCA1* and *LHY* transcripts usually peak at dawn. In spite of this, it has been revealed that *CCA1* and *LHY* both exhibit a potent rise in transcription in response to light (Martinez-Garcia *et al.*, 2000) and, hence, the onset of dawn is also accompanied by an acute spike in *CCA1* transcription (Kim *et al.*, 2003). In summer, the level of the *CCA1* and *LHY* messengers shows an earlier spike due to the longer days, which initiate the advance phase, while a late spike of *CCA1* and *LHY* transcription would occur during shorter days, when the time of dawn is delayed causing the delay phase (Salome and McClung, 2005). It is proposed that *PHYTOCHROME*

INTERACTING FACTOR 3 (PIF3) containing a basic helix loop DNA-binding domain is involved in light regulation of *CCA1* and *LHY* transcription by directly binding to the G-box in their promoter sequences, and interacts with both PHYA and PHYB (Martinez-Garcia *et al.*, 2000). On the contrary, a *PIF3* loss-of-function mutant does not compromise clock function, indicating that *PIF3* may not be involved in the signalling pathways mediation (Viczian *et al.*, 2005). However, accurate correlations between the functioning of the main circadian clock genes and the light-signal transduction pathways are not fully accomplished yet.

Michael *et al.* (2008) believed that the *Arabidopsis* circadian clock has a temperature sensitive oscillator that can be phased distinctly from a light sensitive oscillator. For instance, the phases of *CHLOROPHYLL A_B BINDING PROTEIN 2* (*CAB2*) and *TOC1* expression are altered by light and temperature; whereas the phase of *CATALASE 3* (*CAT3*) expression is more sensitive to changes in temperature than light (Michael *et al.*, 2003b). The dynamic balance between *LHY* and *GI* levels is the key for the effective temperature compensation of the circadian clock at high temperatures, while at low temperature *LHY* seems to be substituted by *CCA1* protein, which increased slightly. In addition to this role in temperature compensation, *GI* also plays a critical role in extending the temperature range over which robust and accurate rhythmicity can be maintained (Gould *et al.*, 2006). Furthermore, the *PRR7* and *PRR9* genes are critical components of a temperature-sensitive circadian system since *prr7* and *prr9* double mutant plants lost the ability to reset the clock in response to temperature entrainment and failed to maintain rhythmicity in the dark (Salome and McClung, 2005). *CCA1/LHY* mRNA levels also increase in *prr7* and *prr9* double mutant plants in response to the increases in temperature relative to wild type levels, thus *PRR7* and *PRR9* play an

important role in adjusting *CCA1* and *LHY* activities in response to ambient temperature (Salome *et al.*, 2010). A recent study has indicated that *CCA1* and *LHY* are involved in *Arabidopsis'* freezing tolerance via regulation of the *C-REPEATBINDING FACTOR* (*CBF*) cold-response pathway by binding to the *CBF1–3* locus and inducing expression of *CBF1, CBF2*, and *CBF3* (Dong *et al.*, 2011).

In 2008, Michael *et al.* showed that the circadian clock is involved in the control of dawn and dusk anticipation, which improves photosynthetic performance and increases fitness. According to Dodd *et al.* (2005), *Arabidopsis* with an accurately functioning clock allows plants to accumulate more chlorophyll, fix more carbon, and grow faster, which leads to improved survival and confers a competitive advantage compared with plants defective in clock function. It has previously been stated that the decreased growth of the *cca1/lhy* double mutant under the daily cycle is a result of exhausting its starch prematurely, leading to a period of C starvation at the end of night (Graf *et al.*, 2010).

This project will match the endogenous clock period with the period of exogenous light: dark cycles in barley in order to study the outcomes. This will be done by comparing the performance indicators such as wet weight, dry weight, leaf area, relative growth rate (RGR), leaf weight ratio (LWR), leaf area ratio (LAR), specific leaf area (SLA) and unit leaf rate (ULR) and chlorophyll content of wild type *PPd-H1* plants with late flowering *Ppd-H1* mutant plants that had a point mutation in the *Ppd-H1* gene in a range of environmental period lengths (T cycle). Barley plants will be grown under 10 hours light-10 hours dark (T20), 12 hours light-12 hours dark (T24) and 15 hours light-15 hours dark (T30) at different temperatures (12°C, 17°C and 27°C). Experiments will be

performed during vegetative growth to determine the implications of circadian resonance on growth and fitness.

The aim of this section is to identify whether accurate and robust clock function is important in barley. It also aims to investigate whether the clock-associated performance reduction is temperature dependent. This will test the hypothesis 'Is clock function an important trait in barley plants?', which might be answered by performing these experiments. Moreover, these experiments may confirm whether the *Ppd-H1* could also be a part of the central clock. As previously reported, *PPd-H1* is a pseudo response regulator and most similar to *AtPRR7*, which is considered part of the morning loop and involved in both the flowering pathway and temperature compensation in *Arabidopsis* (Turner *et al.,* 2005; Locke *et al.,* 2006; Nakamichi *et al.,* 2007; Salome *et al.*, 2010). Alternatively, Cotter (2010) pointed out that *HvPPDH1* is positioned in close proximity to *AtPRR3* on a phylogenetic tree and oscillated in a circadian manner with transcription peaks in the subjective evening. Moreover, he also reported that the *ppd-H1* mutant displayed a shorter period of transcript at 17°C, whereas at 22°C it displayed a long period, and at 27°C the clock genes showed no effect. Therefore, *HvPPDH1* might be a key regulator in temperature sensitivity in barley.

5.2 Results

Initial experiments were conducted to quantify the importance of light and temperature compensation of the circadian clock on the enhancement of growth and fitness in barley plants. The effects of light:dark cycles and temperature on the

performance of three genotypes (Golden Promise, PPd-H1-WT, and Ppd-H1 mutant) were analysed using a set of experiments. The following morphological parameters measured at the end of the experiments, wet and dry shoot weight, plant height, relative growth rate (RGR), leaf weight ratio (LWR), leaf area ratio (LAR), specific leaf area (SLA) and unit leaf rate (ULR). Total amount of chlorophyll content at the end of experiments was also quantified.

Among all measured morphological parameters, a significant difference was only observed between the interaction of light:dark cycles with temperature and genotype in fresh and dry shoot weight, average plant length and chlorophyll content. PPd-H1-WT entrained at constant light conditions with 22°C had the greatest fresh and dry shoot weight (Figure 5.1, A and B). In contrast, the Ppd-H1 mutant had the highest average plant length/height when grown under constant light conditions at 27°C (Figure 5.1, C), as well as chlorophyll content at constant light conditions and 22°C (Figure 5.1, E). Moreover, the morphological parameters showed considerable differences between T cycles ($P < 0.001$). Most of these morphological parameters - fresh and dry shoot weight, average plant length/height, amount of chlorophyll content, RGR and ULR - had the highest values under constant light conditions, except for LAR and SLA, which showed the highest values (186.5 and 444.9, respectively) under T24 (Figure 5.2, B and C); whilst LWR showed no significant differences between constant light conditions, T24 and T30, compared with T20, which showed the lowest value (0.4112) (Appendix 2).

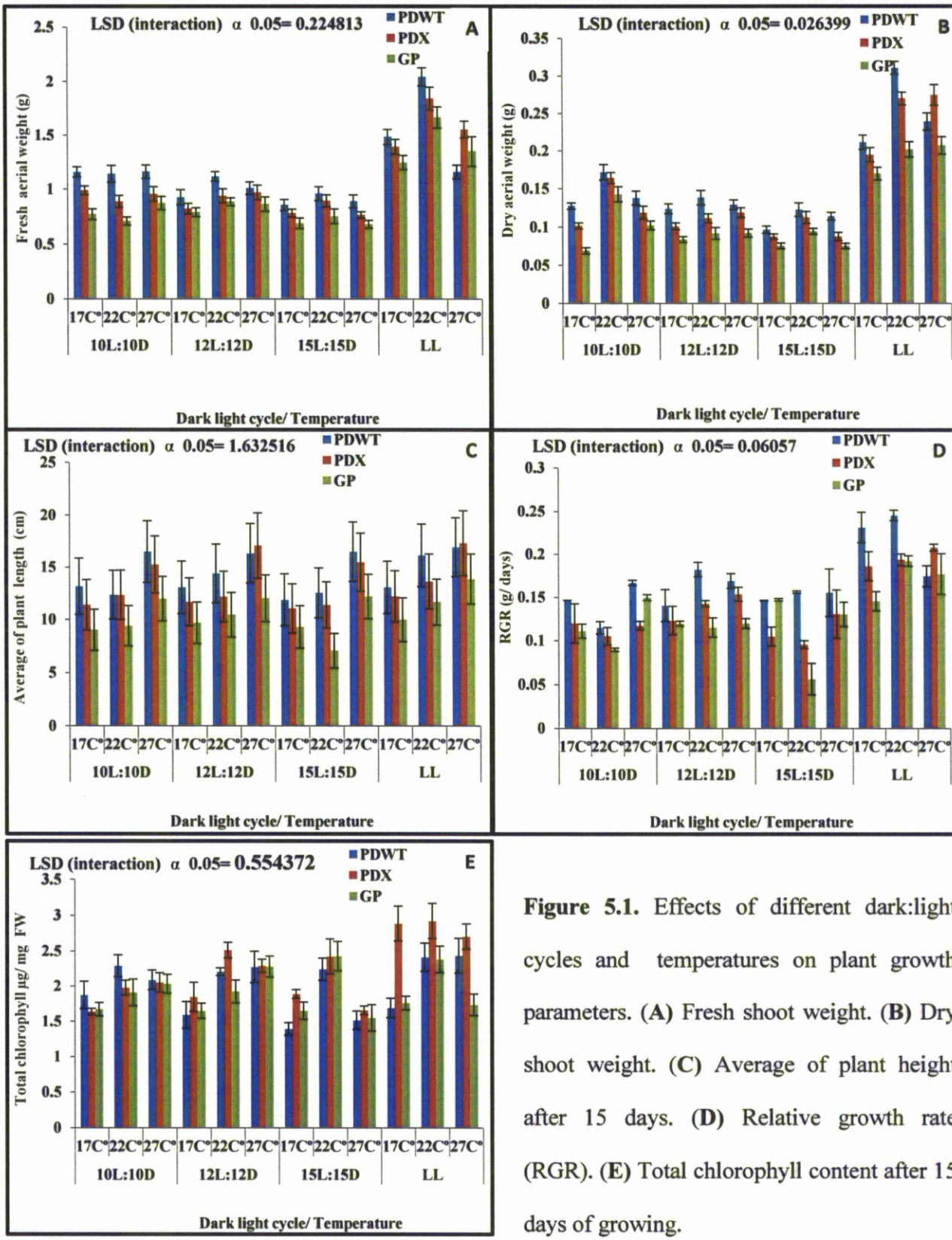


Figure 5.1. Effects of different dark:light cycles and temperatures on plant growth parameters. **(A)** Fresh shoot weight. **(B)** Dry shoot weight. **(C)** Average of plant height after 15 days. **(D)** Relative growth rate (RGR). **(E)** Total chlorophyll content after 15 days of growing.

Across all temperature treatments, fresh and dry shoot weight, chlorophyll content, LAR and SLA had the highest recorded values at 22°C (Figures 5.1A, B, E and 5.2B, C); whilst, for average plant length/height, RGR and LWR had the highest values at 27°C (Figures 5.1C, D and 5.2A). In contrast, ULR exhibited no significant differences between both 17°C and 27°C in comparison with 22°C (Figure 5.2D).

According to the analysis of variances (ANOVAs), interactions between T cycles and temperatures were significantly different ($P < 0.001$) in all morphological parameters. Most of the morphological parameters showed the highest values at 22°C and constant light conditions, except for both average plant length and LWR, which had the highest values at 27°C and constant light conditions. On the contrary, both LAR and SLA were given highest values at 22°C and T24 dark:light cycle (Appendix 2).

Other morphological parameters - fresh and dry shoot weight, RGR and total chlorophyll content - showed slight differences in response of the genotype to the T cycle treatments. Nearly all of the assessed genotypes showed the highest values of fresh and dry shoot weight (Figure 5.1A and B), RGR (Figure 5.1D) and total chlorophyll content (Figure 5.1E) at constant light conditions compared with other T cycles. At the same time, genotype response to the temperature treatments displayed substantial differences on fresh and dry shoot weight, average plant length, LWR and LAR. Almost all of the genotypes gave the highest values of fresh and dry shoot weight, and LAR at 22°C (Figure 5.1A and B and 5.2B), whilst the highest mean values of plant height and LWR were recorded in all genotypes grown at 27°C (Figure 5.1C and 5.2A).

The performance of PPd-H1-WT in most of the treatments was better than that of other lines. However, there are no significant differences between PPd-H1-WT and Ppd-H1 mutant in both parameters LWR and LAR (Figure 5.2A and B). Furthermore, PPd-H1-WT showed the lowest values in both parameters LAR and SLA (147.5 and 327.5, respectively). In sharp contrast, the most interesting findings are that leaf chlorophyll concentration was considerably higher in Ppd-H1 mutant leaves than the other barley lines at constant light conditions (Figure 5.1E); whilst GP tended to give the highest LAR and SLA in the most of the treatments (Figure 5.2C).

Another way of presenting the result from these experiments is a direct comparison among genotypes and T cycle at 22°C. Most of the morphological parameters had slightly differences between T cycle treatments at 22°C. Fresh and dry aerial weight, average plant length, total chlorophyll content, RGR and ULR were given highest values under constant light conditions (Figure 5.3 and 5.4D). On the contrary, LAR and SLA showed highest values under T24 (Figure 5.4 B and C), while there is no significant difference has been pronounced between T cycle in LWR (Figure 5.4 A). Moreover, Ppd-H1-WT had greatest fresh and dry aerial weight, average plant length and RGR at 22°C (Figure 5.3). Alternatively, Ppd-H1 mutant estimated highest values of total chlorophyll content (Figure 5.3). Also no significant differences were observed in LWR, LAR, SLA and ULR between genotypes at 22°C (Figure 5.4).

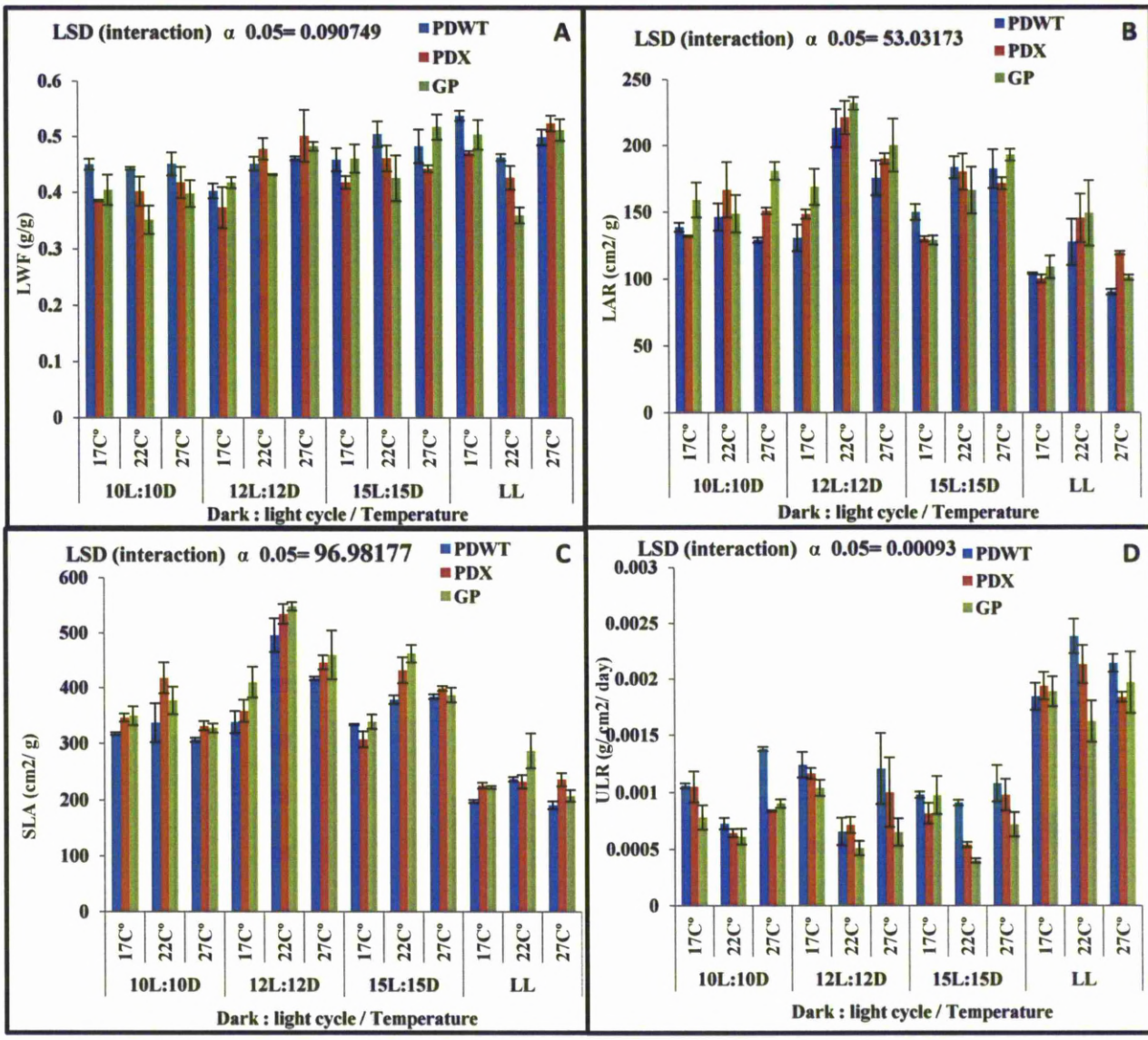


Figure 5.2. Effects of different dark: light cycles and temperatures on plant growth parameters. **(A)** Leaf weight ratio (LWR). **(B)** Leaf area ratio (LAR). **(C)** Specific leaf area (SAL). **(D)** Unit leaf rate (ULR).

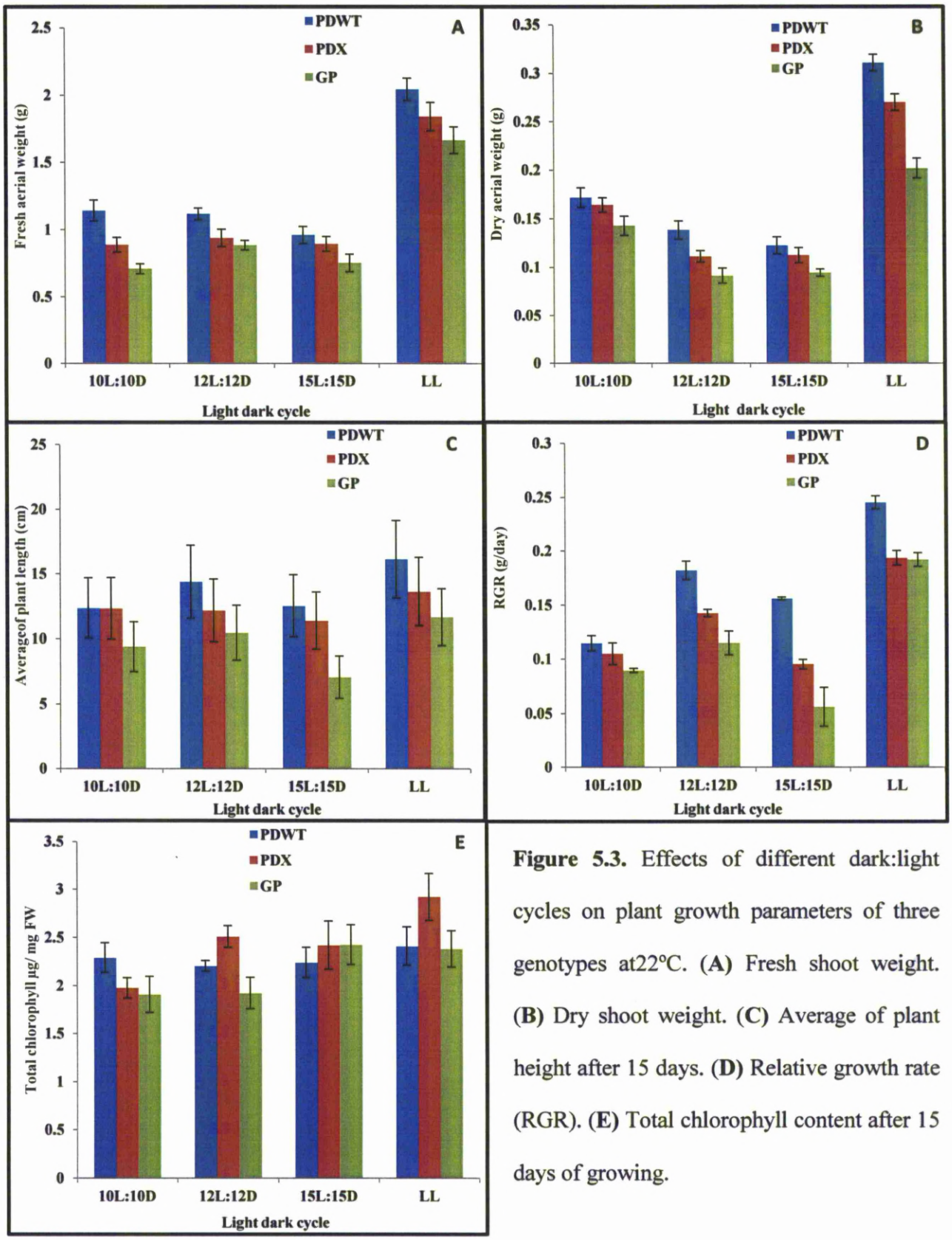


Figure 5.3. Effects of different dark:light cycles on plant growth parameters of three genotypes at22°C. **(A)** Fresh shoot weight. **(B)** Dry shoot weight. **(C)** Average of plant height after 15 days. **(D)** Relative growth rate (RGR). **(E)** Total chlorophyll content after 15 days of growing.

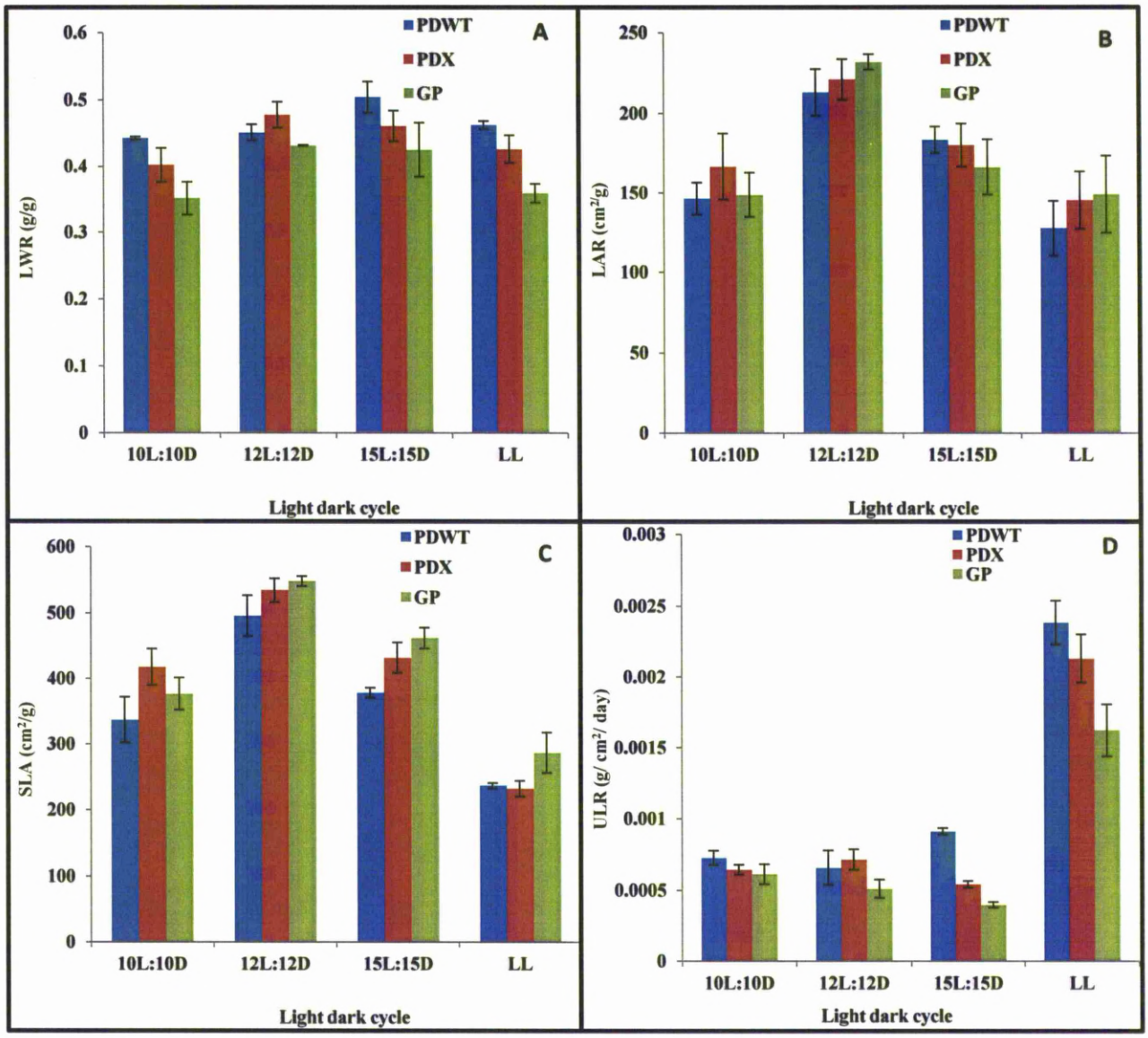


Figure 5.4. Effects of different dark: light cycles on plant growth parameters of three genotypes at 22°C. **(A)** Leaf weight ratio (LWR). **(B)** Leaf area ratio (LAR). **(C)** Specific leaf area (SAL). **(D)** Unit leaf rate (ULR).

5.3 Discussion

It was discovered that *Arabidopsis* plants with an endogenous circadian system that resonates with its environmental changes might confer an adaptive and fitness advantage. However, it has not been possible to illustrate this potential advantage in other plant species yet. This chapter has provided a brief introduction to understanding the influence of light-dark cycles and temperature on growth adaptation and fitness of barley plants. A set of experiments was performed to examine the effect of T cycles and temperature on the endogenous clock period of three genotypes (Golden Promise, PPd-H1-WT, and Ppd-H1 mutant), and also to determine if the *Ppd-H1* is a clock gene. Statistically, a general linear model (GLM) analysis was used to establish a comparison between and within treatments at $P \leq 0.05$.

The results of the GLM analysis have shown that the performance of the three genotypes was significantly ($P \leq 0.05$) affected by T cycle and temperature experimental conditions. The three cultivars showed higher values in the morphological growth parameters measured under constant light condition including: fresh and dry shoot weight, average plant height, amount of chlorophyll contents, RGR and ULR. It has been revealed that continuous light is a useful tool to investigate and characterize the plant circadian clock and its regulated processes such as temperature compensation (Velez-Ramirez *et al.*, 2011). These findings are consistent with other studies described by Sysoeva *et al.* (2010). It can be clearly confirmed that the increase of the light period led to an increase in the total chlorophyll content per unit leaf area, which may be due to increased carbon fixation in the plant, hence increasing the plant growth rate and fresh and dry shoot weight.

Relative growth rate (RGR) is influenced by SLA, LWR and ULR. Under constant light conditions, the stimulation of RGR was mainly caused by increasing the ULR rather than increases in SLA. The statistical analysis revealed that LWR is unaffected by the changes in day length between constant light conditions, T24 and T30, compared with T20, which gave the lowest values in most of the genotypes. LAR is determined by LWR and SLA. It seems that the higher LAR at T24 was generally as a result of increased SLA. In general, increased ULR is frequently associated with a reduced level of SLA. ULR is an expression of the increase of dry matter per unit leaf area and is a measure of the excess of photosynthesis over respiration, which demonstrates the optical efficiency of the leaf in dry matter production (Thorne, 1974); while the SAL is a reflection of the leaf thickness and leaf density, which is dependent on the amount of air space inside the leaf tissue and the amount of water per dry mass (Witkowski and Lamont, 1991). Growing barley plants under constant light conditions might lead to the production of thicker leaves with low SLA owing to the stimulation of extra layers of palisade cells those likely causes increasing chloroplast number and amount of photosynthesis capacity per unit leaf area.

The maximum rates of plant growth in the three barley genotypes occurred at 22°C, with a sharp decline on either side of this temperature. The data show that increasing the temperature from 17°C to 22°C led to an increase in both fresh and dry shoot weight, but this expansion declined at 27°C. A possible explanation of increasing shoot weight with the increase of temperature from 17°C to 22°C could be that the increase of temperature stimulating the leaf area production (SLA), rather than an effect of photosynthesis per unit of leaf (ULR). However, the reduction in both fresh and dry shoot weight by increasing the temperature from 22°C to 27°C might be due to the higher rate of plant respiration and lack of CO_2 absorption and general stress. At 27°C, all barley genotypes

reached maximum plant length and highest LWR. It is more likely that increasing the temperature led to an increase in stems and leaf formation by enhancing the length of both stem cells and leaf blades.

One aim of running these experiments was to confirm whether the Ppd-H1 is a clock gene. The Ppd-H1 mutant has a late flowering phenotype and, overall, is most similar to *Arabidopsis PRR7* mutants that display delayed flowering in LDs (Turner *et al.*, 2005). According to Dodd *et al.* (2005), *Arabidopsis* with an internal period matched to the environment accumulated more chlorophyll, fixed more carbon and grew faster, which led to improved survival and conferred a competitive advantage. A short period (*toc1-1*) and a long period (*ztl-1*) mutants displayed growth enhancement over wild type plants (*Col-0*) when they were grown under photoperiod T20 and T28 respectively. Likewise, when wild type plants (*Col-0*) were grown in light periods (T24) they exhibited higher growth parameters over *toc1-1*, *ztl-1* and arrhythmic mutant (*cca1-ox*) (Dodd *et al.*, 2005). Furthermore, it has been observed that the long hypocotyls phenotype of circadian clock mutants (*elf3, elf4, lux, lux-2* and *lhy*) exhibited under dark:light cycles can be rescued under constant light (Michael *et al.*, 2008). By performing experiments similar to those described by Dodd et al. (2005), it would be easy to distinguish the differences in performance of both genotypes PPd-H1-wt and Ppd-H1 mutant under different environmental conditions. However, the current study was unable to confirm if the Ppd-H1 mutant is a clock mutant, due to the limited amount of time available to conduct experiments until plants became fully mature.

It would appear that the difference in response of the three genotypes to the range of photoperiods and temperatures tested was not significant. Overall, it seems that all genotypes widely adapted to the changes in both photoperiod and temperature. *PPd-H1*

wild type had the highest growth parameters, except for LAR and SLA, across a broad range of environments. This finding further supports the hypothesis of the late flowering ppd-H1 allele extending the vegetative growth period (Wang *et al.*, 2009). Another important finding is that the Ppd-H1 mutant gave the highest value of leaf chlorophyll concentration at the end of the experiments than the genotypes under constant light conditions. According to Fukushima *et al.* (2009), *PRR9/7/5* negatively regulates the biosynthetic pathways associated with chlorophyll, carotenoid-ABA and α-tocopherol biosynthesis in chloroplasts. The Ppd-H1 mutant might be adapted to constant light by increasing the amount of chlorophyll. This finding raises the possibility that the Ppd-H1 mutant may actually be a clock mutant altering the biology of a sub-set of circadian regulated responses.

Conducting these experiments has provided insight into the impact of the light:dark cycle and temperatures on growth and adaptation of barley. This study confirms that the *PPd-H1* wild type widely adapted to the changes in the environment and gave the highest growth parameters at constant light, except for LAR and SLA, which had highest values at T24. However, the findings of the current study do not support the earlier findings by Dodd *et al.* (2005); they reported that wild type plants (*Col-0*) grown in light periods (T24) exhibited higher growth parameters over *toc1-1*, *ztl-1* and arrhythmic mutant (*cca1-ox*), while the short period (*toc1-1*) and long period (*ztl-1*) mutants displayed growth enhancement over wild type plants (*Col-0*) when they were grown under photoperiods T20 and T28.

CHAPTER 6: DEVELOPING A CLOCK ASSAYING FOR BARLEY PLANTS USING DELAYED FLUORESCENCE

6.1. Introduction

Delayed fluorescence (DF) may be described as light emission of photosynthetic organisms shortly after their source of illumination has stopped; it was discovered accidentally by Strehler and Arnold (1951). DF results from the post-illumination emission of light from chlorophyll, essentially from photosystem II (PSII), are as a consequence of charged recombination between excited plastoquinone QA and P680 leading to the emission of a photon (Gould *et al.*, 2009). The level of DF is under robust circadian control and oscillates around the 24-h period in a variety of plants such as *Arabidopsis*, *K. Fedtschenkoi* and *Zea mays* (Gould *et al.*, 2009). There is apparent evidence in several species that CO_2 assimilation and light-induced electron flow are clock controlled (Samuelsson *et al.*, 1983; Hennessey *et al.*, 1991). There was also a remarkable correlation between the period length of the *K. fedtschenkoi* DF rhythm and the period of the CO_2 fixation rhythm of equivalent leaves (Gould *et al.*, 2009). In *Arabidopsis*, many of the key genes that constitute the light harvesting complex and PSI and II are under circadian control at the level of the associated steady-state transcript abundance (Gould *et al.*, 2009). In addition, observation of Chloroplast Gene Expression in *Chlamydomonas reinhardtiiusin gpsbD-lucCP* bioluminescence reporter suggested that the period length of the chloroplast rhythm was related to the nucleus-encoded circadian oscillator (Matsuo *et al.*, 2006).

The DF technique may provide a simple and reliable method for assaying the clock output in any photosynthetic organism. It does not require the insertion of a transgene,

thus less influence on the environment. It also could be detected by using existing lens-based CCD imaging systems that are currently used to measure luciferase activity (Gould et al., 2009). It also allows accurate measurement of phase as well as of the period. The main advantage of the DF imaging is that the DF could be potentially useful for reducing the time needed for screening of mutants. It could also be used in a breeding programme, allowing rapid phenotypic analysis of the circadian clock

6.2. Results

A protocol has been developed to measure robust DF rhythms in barley plants. The method was originally development by Gould et al. (2009) to measure the robust circadian clock in *Arabidopsis*. In this chapter the protocol from *Arabidopsis* has been adapted to suit the physiology of the barley plant and used to confirm whether the *HvPPDH1*gene is the barley *PRR7* gene, and also uncover if the *ppd-H1* mutant is a clock mutant.

6.2.1. Measurement of DF in barley plants

DF may provide a universal method for measurement of circadian rhythms and automated screening of the interesting mutants in barley plants. In order to test circadian rhythms, barley plants (Golden Promise) were grown in 12:12 h light:dark cycles at 22°C conditions. Leaves were cut into 1 cm pieces and placed in 25 compartmental square Petri dish plates and floated on SDW. The leaves were exposed to 1 min constant RB light (35μmol $m^{-2}sec^{-1}$) every one hour after switching the LED light off. This fully automated process was repeated every 1 hour for a period of 96 h. The initial results indicated that

DF did not show the regular rhythms (Figure 6.1A) owing to sealing the side of the plates with tape, which is likely to be because of the loss of oscillatory behaviour of the DF. The same experiment was repeated by leaving the side of the plate slightly open. The tissue culture plates have two positions. One position allows the gas flow in and out of the plates, while the second position seals the plates completely and prevents the gas flow. The second experiment was run with the first position of the plates, allowing the air flow around the cut leaves. The oscillation pattern of the DF showed substantial improvement (Figure 6.1B) in comparison with the first experiment's data. These observations confirm that the magnitude of the DF signal is influenced by exchange of gases around the barley leaves.

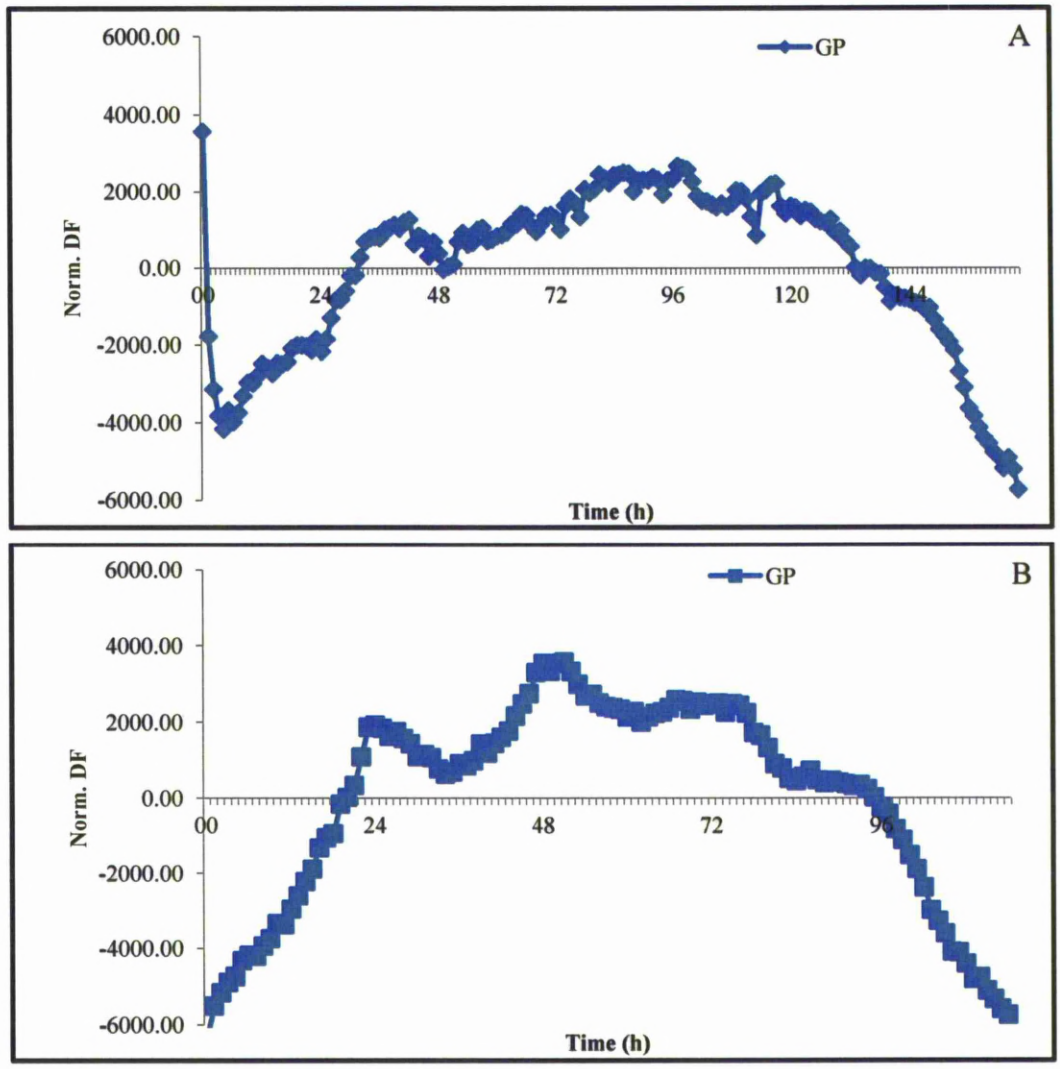


Figure 6.1. Normalized averages of the DF rhythms of 25 Golden Promise cuttings of barley leaves. **A,** without gas exchanges; **B,** with gas exchanges.

6.2.2. Optimization of leaf age

In order to confirm whether the DF rhythms can be truly reflected by the leaf age, or the adaxial and abaxial leaf surfaces of the first or second leaf blade, Golden Promise barley seeds were grown in 12:12 h light:dark cycles at 22°C for one, two and three weeks in the plant growth room. A number of leaves from the first and second leaf blades were cut into 1cm pieces and placed in Petri dish plates containing SDW and the adaxial or abaxial leaf surfaces were exposed for 1 min to constant RB light (40 μmol m^{-2} sec^{-1}) every one hour after switching the LED light off. The results demonstrated that the amount of DF oscillation was considerably greater in barley leaves up to 3 weeks old. However, it was found that using leaves more than 3 weeks old meant that DF rhythms were either unlikely to be detected, or that they dampened after a couple of days. Figure 6.2 shows that the most pronounced differences between the amounts of DF oscillate from leaves of diverse age. At the same time, no significant differences were recorded between exposing the adaxial and abaxial leaf surfaces to the light signal or by using first and second leaf blades (Figure 6.3).

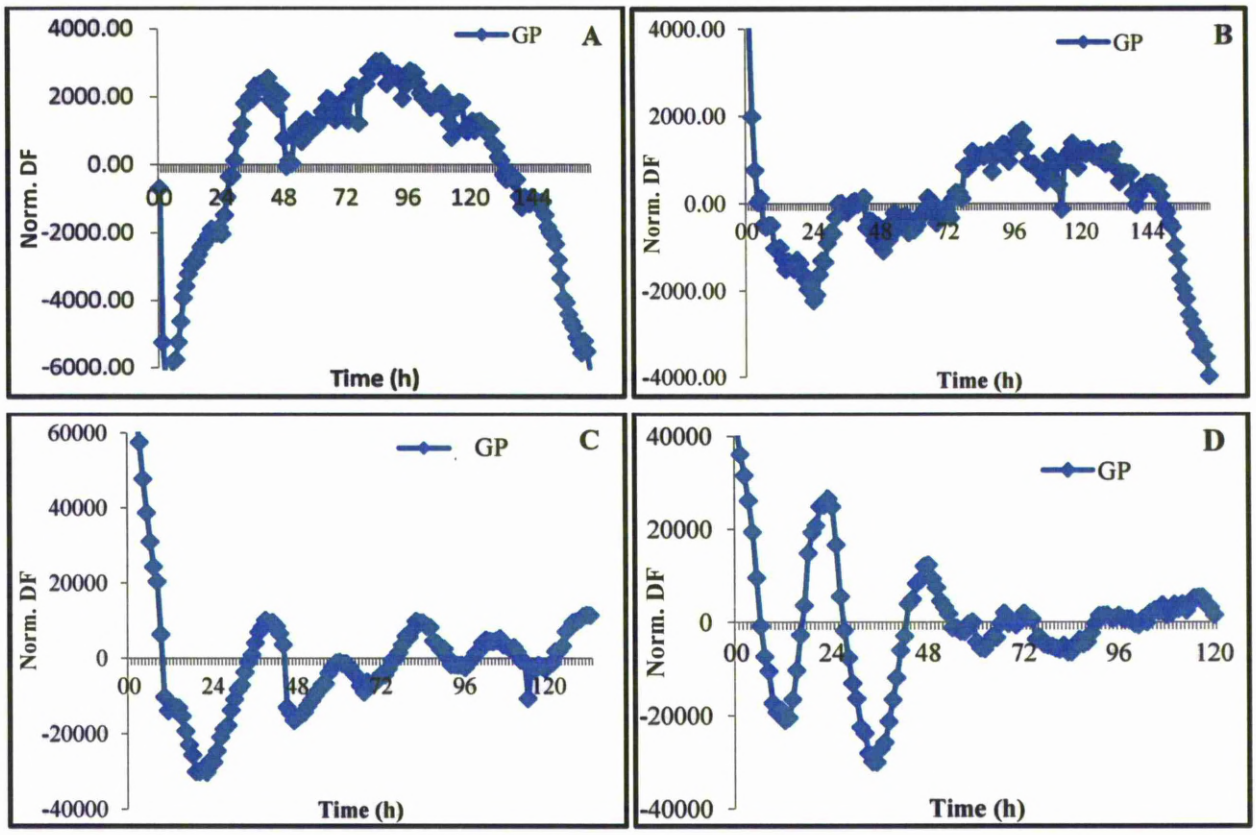


Figure 6.2. Normalized averages of DF rhythms of 25 Golden Promise cuttings of barley leaves. **(A)**, one-week-old plant; **(B)** two-week-old plant; **(C)** three-week-old plant; and **(D)** four-week-old plant.

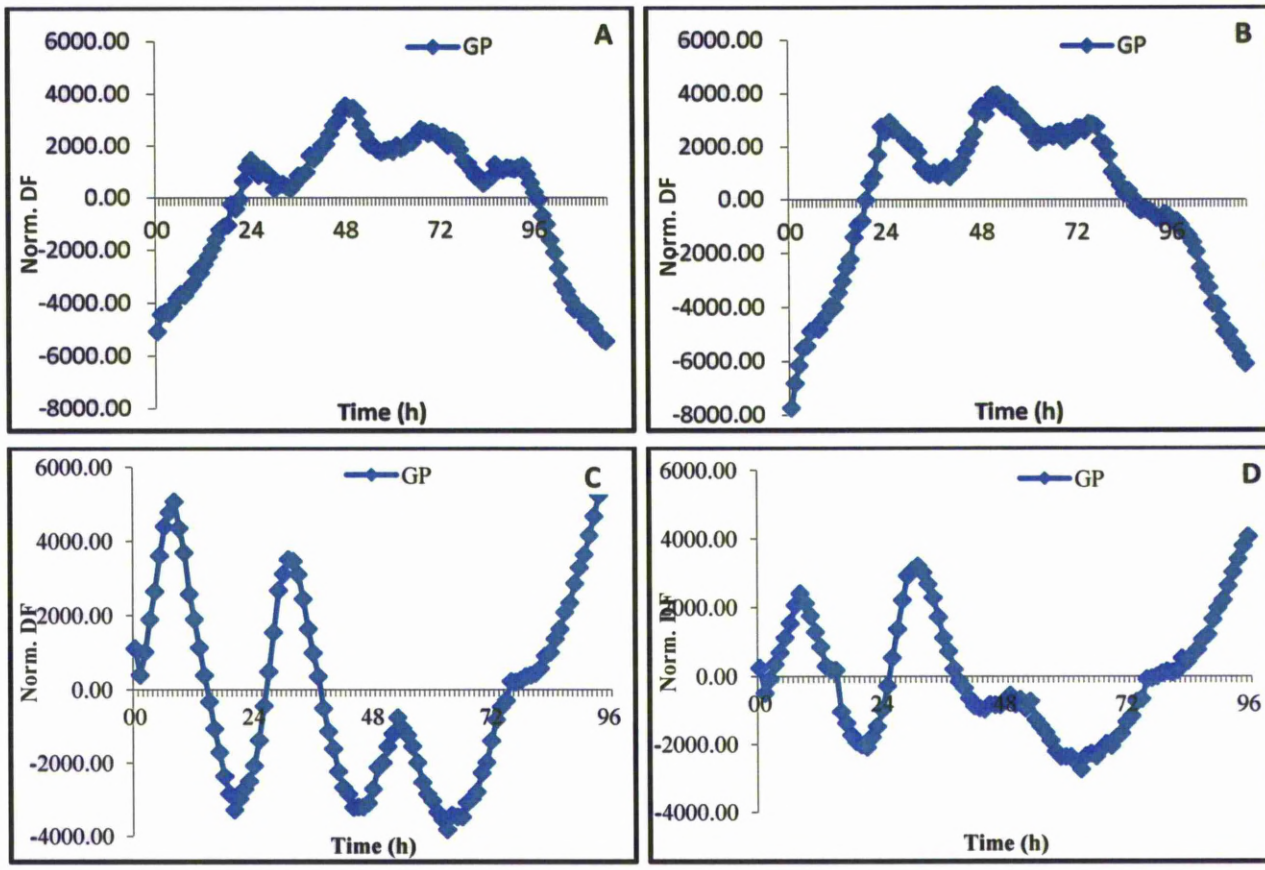


Figure 6.3. Normalized averages of the DF rhythms of 25 Golden Promise cuttings of barley leaves. **(A)** Abaxial leaf surface. **(B)** Adaxial leaf surface. **(C)** First leaf. **(D)** Second leaf.

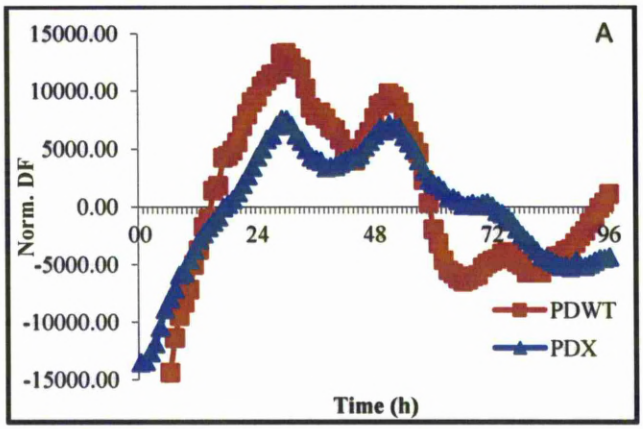
6.2.3. Optimization of light level

The next challenge addressed in this research was that of using the DF technique to monitor the circadian clock and automated screening of the interesting mutants in a diverse range of barley genotypes. In the following experiments, the DF luminescence was measured in two barley genotypes (PPd-H1wild type and Ppd-H1 mutant) following the same procedures described above. At the same time, the DF experiments were run under different ranges of constant RB light intensity (140, 110, 80, 40 and 10μ mol m^{-2} sec^{-1}) to optimize the appropriate light intensity. The results obtained from these experiments will confirm whether the *Ppd-H1* and *HvPPR7* genes are similar, as well as investigating the influence of light intensity on the amount of DF rhythms. Turner *et al.* (2005) indicated that there is a high similarity between *Ppd-H1* and *Arabidopsis PPR7* genes.

The most interesting results were obtained when the intensity of light was increased to 140 μ mol m^{-2} sec^{-1}. At this intensity, the amount of DF rhythms was considerably increased (Figure 6.4 A) but these DF rhythms decreased rapidly within a couple of days due to early leaf senescence; this might be caused by exposing barley genotypes to a high level of light intensity. Somehow, it is difficult to sustain the cut barley leaves immersed in water for long periods without the addition of some nutrients. Efforts were made to maintain the robust rhythms for a period of time (5 days) in constant light conditions to avoid early leaf senescence by decreasing the light intensity to 30 μ mol m^{-2} sec^{-1}. The results demonstrated that dropping the light intensity to a low level caused a slight decline in the oscillation of the DF (Figure 6.4).

On the other hand, by reducing light intensity to 40μmol m^{-2} sec-1, the early leaf shrivelling/dropping was eliminated and it was possible to obtain the visualisation of

circadian rhythms for a prolonged amount of time. However, decreasing the light intensity to less than 40μ mol m^{-2} sec^{-1} seemed to cause the loss of oscillatory behaviour of the DF (Figure 6.4). The current observation suggested that placing cut leaves under 40 μ mol m^{-2} sec^{-1} constant light is likely capable of preserving the circadian rhythms for 5 days with a good separation of DF oscillation period and pattern compared with the data obtained by using light levels of 110, 80, and 10 μ mol m^{-2} sec^{-1} (Figure 6.4). The data presented from these experiments suggest that it is not possible to use DF for assaying the barley clock of the genotypes or screening for the existing clock mutation. However, these results provided an overview of the influence of the light level in barley DF oscillation.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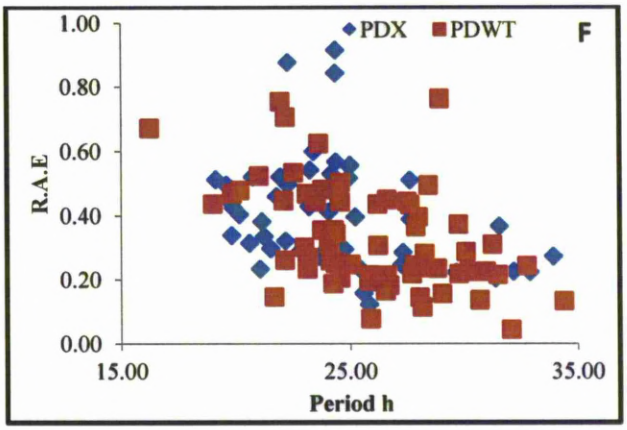
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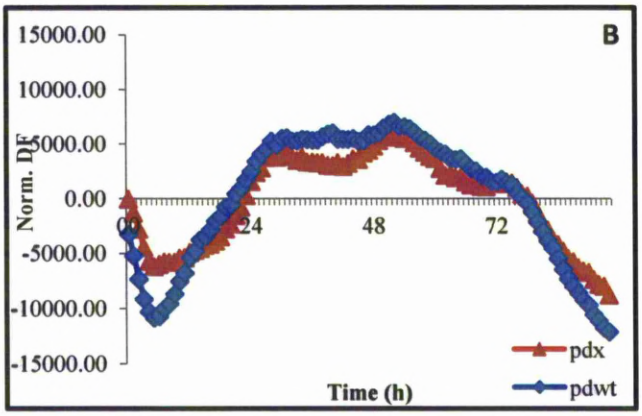
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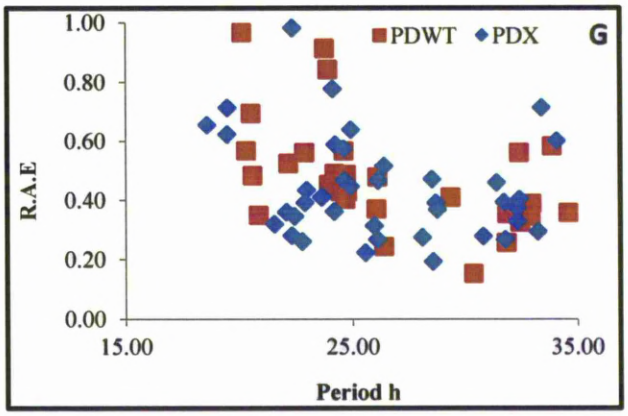
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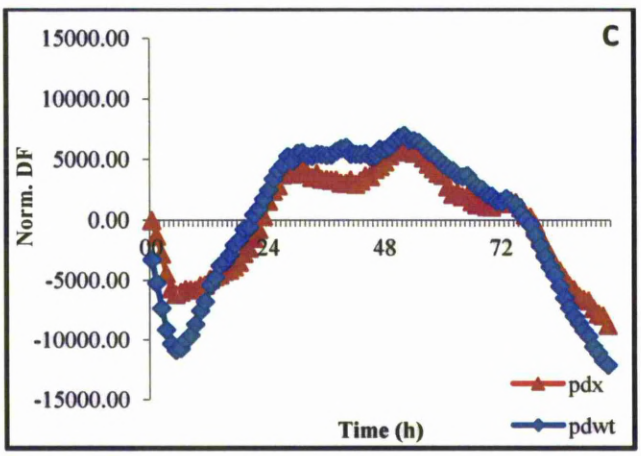
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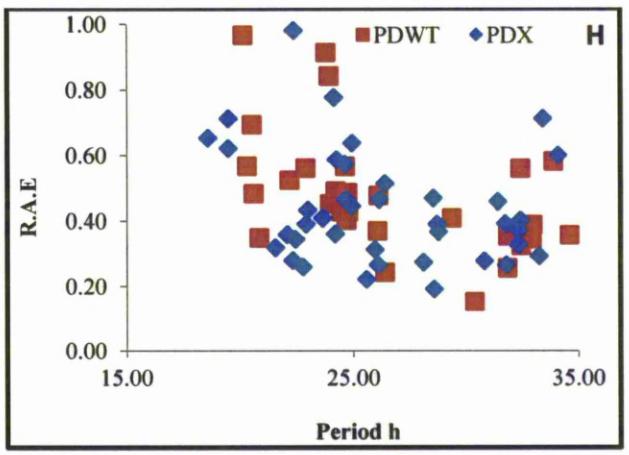
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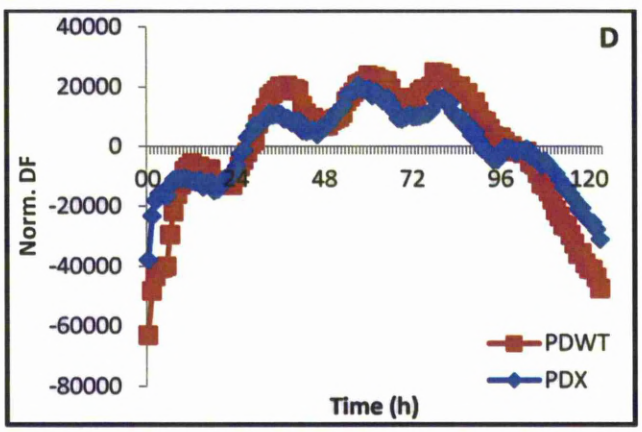
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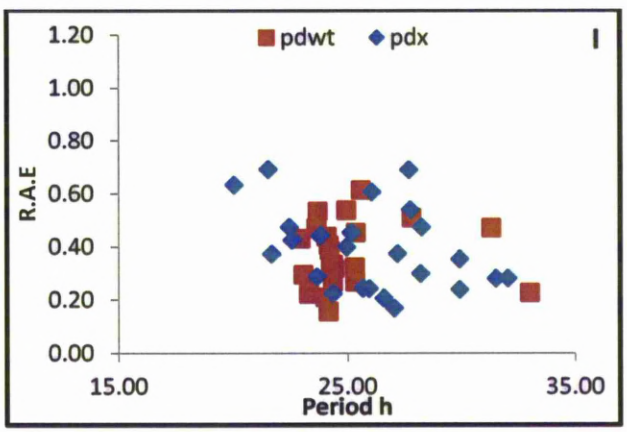
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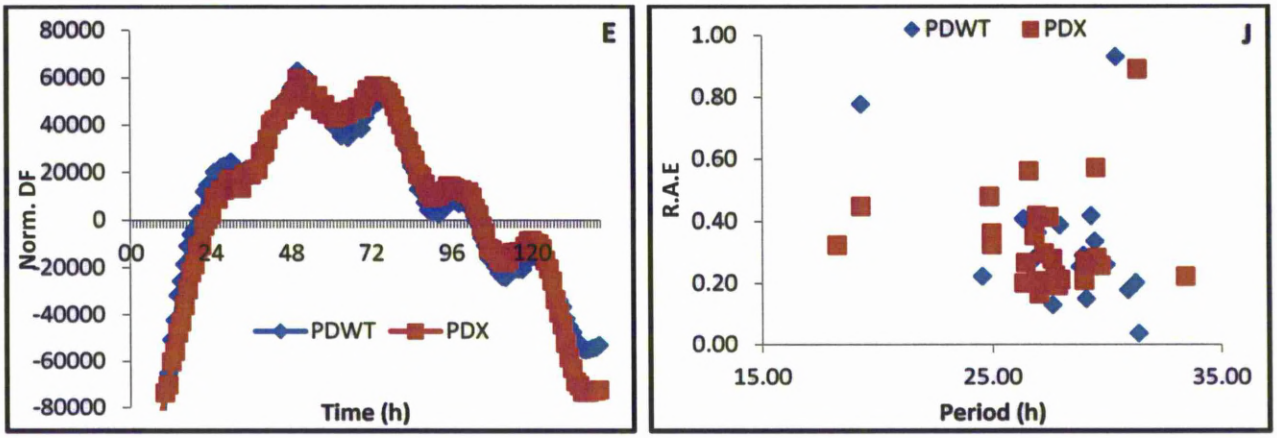

Figure 6.4. Measurement of delayed fluorescence rhythms of two barley genotypes, PPd-H1 wild type and Ppd-H1 mutant, at different light levels. (**A and E**) Normalized averages for DF rhythms of 25 leaf cuttings of two barley genotypes. (**F and J**) DF period plotted against their RAE estimated from leaves of two barley genotypes. (**A and F**) RB light 140μmol m^{-2} sec^{-1}; (**B and G**) RB light 110 μmol m^{-2} sec^{-1}; (**C and H**) RB light 80 μmol m^{-2} sec^{-1}; (**D and I**) RB light 40 μmol m^{-2} sec^{-1}; (**E and J**) RB light 10 μmol m^{-2} sec^{-1}.

6.2.4. Entrained barley plants at long and short days

In order to enhance the DF rhythm's pattern and avoid early leaf shrivel/drop, in the following experiments the two barley genotypes were grown under different environments. part of the barley pots were grown in a greenhouse under high intensity light and others were incubated in a plant growth chamber under long days (16:8 h light:dark cycles) and short days (8:16 h light:dark cycles) at 22°C with a light level over 500 μmol m^{-2} sec^{-1} for three weeks. The cut leaves from each treatment were exposed for 1min to constant RB 40 μmol m^{-2} sec^{-1} and DF was assayed as described before. The data presented here indicated that there is a massive improvement in the pattern of DF oscillation in both barley genotypes when they were previously entrained at high light (greenhouse) or under long days (Figure 6.5). However, neither genotype displayed real

amplitude (Figure 6.5) as a result of early leaf withering/shrivelling. The obtained data from entraining the barley plants in short days seems to be in line with results obtained before when entraining the barley in 12h light:dark cycles and exposing them for 1 min to constant RB 40 μmol m^{-2} sec^{-1} (Figure 6.5). Although there was a high similarity in the pattern of DF oscillation, the real amplitude of both barley genotypes entrained in long days seems to be acceptable compared with barley plants that were entrained in high light or in short days (Figure 6.5).

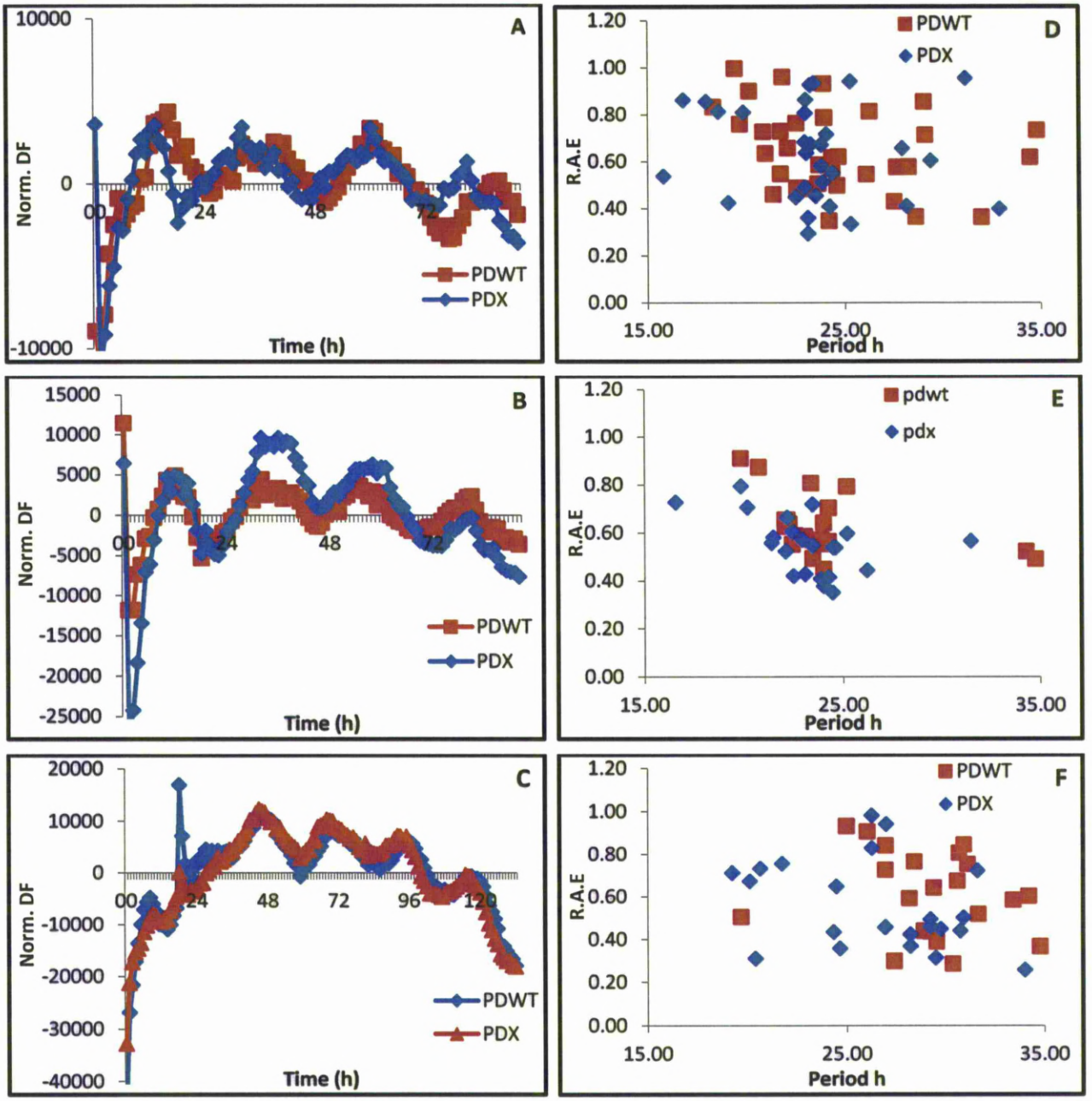

Figure 6.5. Measurements of delayed fluorescence rhythms of two barley genotypes, PPd-H1 wild type and Ppd-H1 mutant, have been entrained under different environments. (**A and C**) Normalized averages for DF rhythms of 25 leaf cuttings of two barley genotypes. (**D and F**) DF period plotted against their RAE estimated from leaves of two barley genotypes. (A and D) Barley leaves entrained in a greenhouse and exposed to RB light 40 µmol m^{-2} sec^{-1}. (**B and E**) Barley leaves entrained in long days (16:8 h light:dark) and exposed to RB light 40 µmol m^{-2} sec^{-1}; (**C and F**) Barley leaves entrained in short days (8:16 h light:dark) and exposed to RB light 40 µmol m^{-2} sec^{-1}.

6.2.5. Measuring DF in constant darkness

The conclusions from the above experiments are that the pattern of DF oscillation was shown to be substantially improved by entraining the barley plants in long days and high light (>500 μmol m^{-2} sec^{-1}) conditions for three weeks. However, maintenance of robust of DF rhythms that were perceptible for a period of time (3-5 days) in constant light conditions proved difficult. Hence, the assay was progressed in barley in order to attempt the visualisation of circadian rhythms in cut barley leaves that was detectable for a longer period of time. In the next experiments, the cut barley leaves of both genotypes, that were entrained in long days and high light (>500 μmol m^{-2} sec^{-1}) at 22°C conditions for three weeks, were placed in constant darkness at 22°C and given pulses of light (40 μmol m^{-2} sec^{-1} RB) for 3 minutes, and a photograph was then taken. In other words, this experiment was run in constant darkness and then the light was switched on for 3 mins before taking the photograph, compared with other experiments that were run under constant light for 59 mins and the light was switched off for 1 min to take a 1 min exposure. There are no differences between the two genotypes and each of them has 25 periods. The results obtained from running the experiments in the dark conditions confirmed that the robustness of bioluminescence rhythms persisted for up to 96 hours, creating a proper protocol for analysis of circadian rhythms in barley plants (Figure 6.6A, B).

6.2.6. Is the *Ppd-H1* gene orthologous to *PRR7?*

The DF results showed that the *Ppd-H1* mutant line exhibited robust DF rhythms that resembled the *PPd-H1* wild type DF rhythms (Figure 6.6A and B). Both lines displayed DF rhythms that peaked at 23, 46 and 70 hours of constant darkness (Figure 6.6A). In PPd-H1 wild types, the average circadian period estimated for DF was 25.41± 0.29 hrs

and RAE 0.36 ± 0.03, while the DF circadian period of the *Ppd-H1* mutant was 25.62± 0.35 hrs and RAE 0.27± 0.03 (Figure 6.6B). This suggests that the point mutation in the *Ppd-H1* gene does not affect the rhythms of DF in constant dark at 22°C. However, Cotter (2010) illustrated that the *Ppd-H1* mutant peaks 1 hour after the wild type DF rhythm when the plates were placed under an imaging camera incubated at 17°C and in conditions of constant darkness. The wild type displayed DF rhythms that peak at 27, 48 and 69 hours in the constant darkness, whereas the *Ppd-h1* mutant DF rhythms peak at 28, 50 and 72 hours. This indicates that the *Ppd-H1* mutant has a period which is longer by 1 hour every cycle in comparison with the wild type. A possible explanation for this is that DF robust rhythms of *PPd-H1* gene might be temperature dependent.

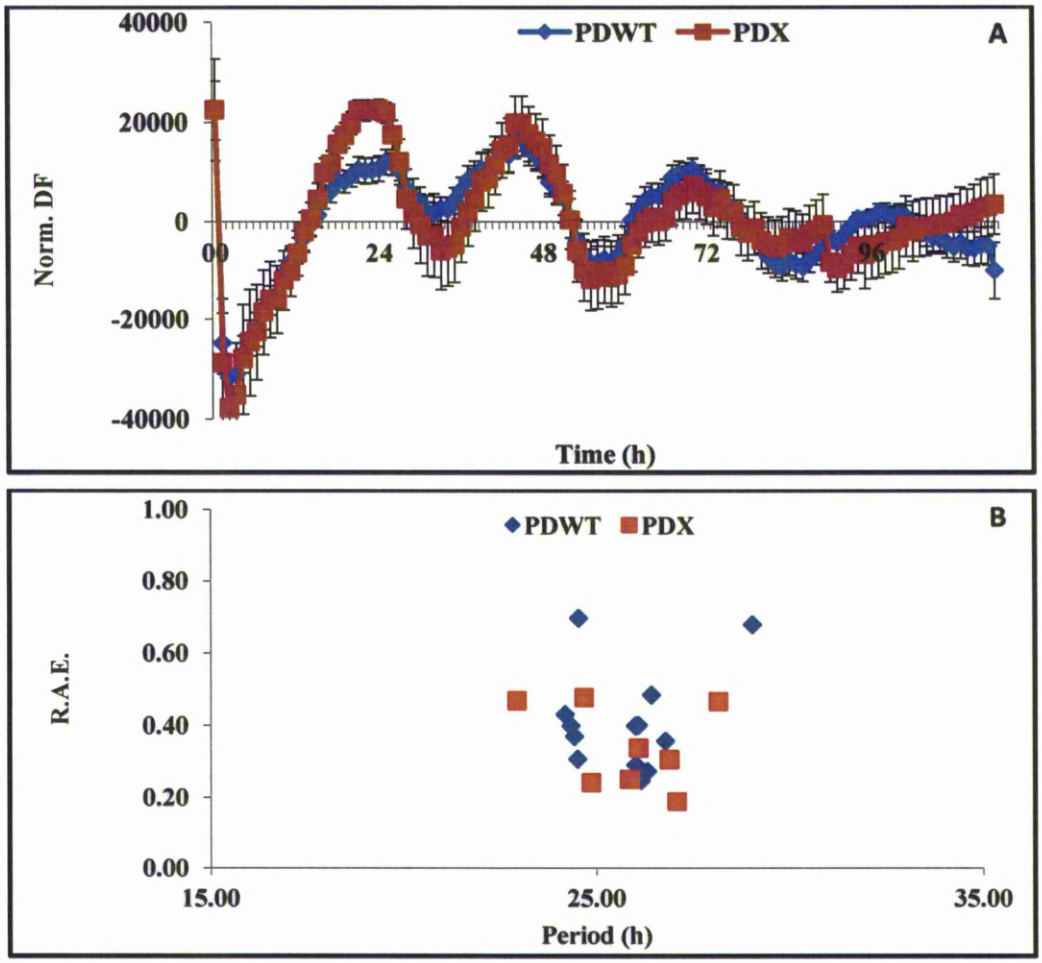

Figure 6.6. Measurement for delayed fluorescence rhythms of two barley genotypes, PPd-H1 wild type and Ppd-H1 mutant, at constant darkness conditions. **(A)** Normalized averages for DF rhythms of 12 leaf cuttings of 2 barley genotypes. **(B)** DF period plotted against their RAE estimated from leaves of 2 barley genotypes. The plant were entrained in long days (16:8 h light:dark) with high light (>500 µmol m^{-2} sec^{-1}) for three weeks. The cut leaves were placed in constant darkness and exposed for 3 mins to RB light 40 µmol m^{-2} sec^{-1} every one hour.

In order to confirm whether the *Ppd-H1* is the barley *PPR7* gene, DF rhythms of PPd-H1 wild type and Ppd-H1 mutant were compared with transgenic lines over-expression *PPd-H1* in Golden Promise barley that was provided by Dr David Laurie. The *PPd-H1* transcript was set under the control of a maize Ubiquitin promoter to guarantee constitutive expression. Because the lines were newly produced and still under segregation, DNA was isolated from five plants of each family (1a and 7a) and a PCR test was run using Hygromycin primers (Hyg F-ACTCACCGCGACGTCTGTC, R-GCGCGTCTGCTGCTCCAT). Then the plants exhibiting the present of a 917bp Hgy band were used to run DF experiments (Figure 6.7). The plants were then transferred to the plant growth room and entrained in 12 h light: dark cycles at 22°C for 3 weeks and DF experiments were run in the constant darkness at 22°C, as described before. The DF results showed that the *ppd-h1-ox* line 7a-4 exhibited robust DF rhythms that were almost equivalent to the wild type GP and both genotypes PPd-H1 wild type and mutant (Figure 6.8A); while the DF rhythms of ppd-h1-ox line 1a-3 peaked 4 hours later in comparison with the DF rhythms of GP, PPd-H1 wild type and mutant. The ppd-h1-ox line 7a-4, GP, PPd-H1 wild type and mutant displayed DF rhythms that peaked at 22, 46 and 70 h in constant darkness. The *ppd-h1-ox line 1a-3* DF rhythms peaked at 26, 50 and 72 hours. However, both of the ppd-h1-ox lines showed a period shorter by almost 1 hour in comparison to the PPd-H1 wild type and mutant lines and longer by approximately 2 hours than wild type GP (Figure 6.8B). The average DF estimated circadian period for ppd-h1-ox line 7a-4 and 1a-3 were 24.47± 0.72 and 24.44± 0.37 hrs, respectively, while the average DF circadian periods of GP, PPd-H1 wild type and mutant were 22.85± 0.56, 24.58± 0.42 and 25.55± 0.37 hrs, respectively (Figure 6.8B).

The DF results support the theory that *ppd-H1* is a clock gene and it is most likely analogous to *AtPRR3*. Although the ppd-h1-ox lines are still under segregation, they displayed a period longer by almost 2 hours than wild type GP. Furthermore, one of the lines exhibited a phenotype of phase delay by 4 hours compared to GP, PPd-H1 wild type and mutant. The DF results from the previous and this experiment revealed that PPd-H1 may be a clock gene and the *ppd-H1* could be also a weak clock mutant with a period longer by 0.5 to 1 hour than wild type and its DF robust rhythms might depend on temperature.

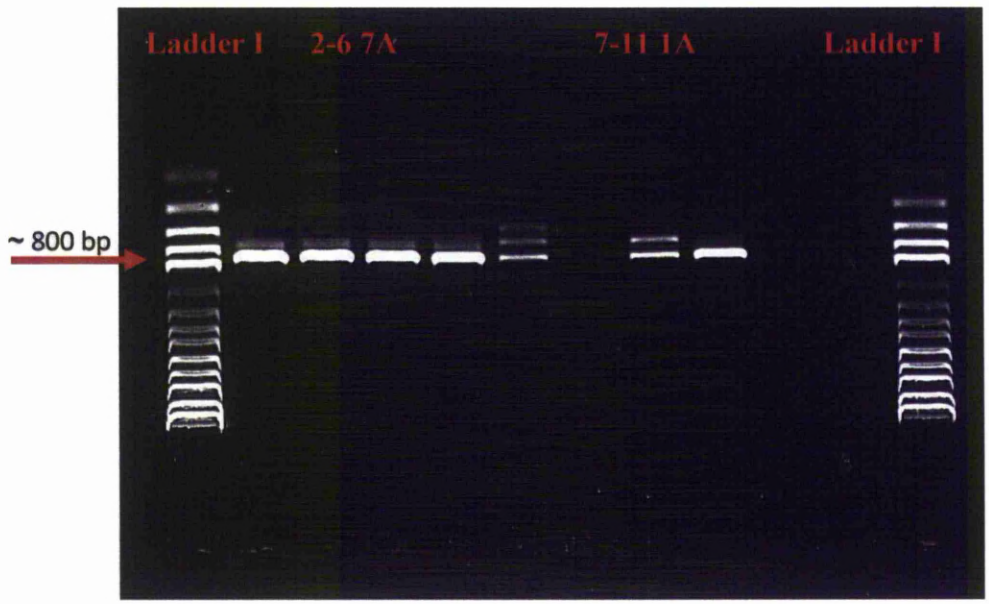

Figure 6.7. 917bp PCR fragments of the hpt gene were amplified from five lines of Ubi-Ppd-H1-Ox using Hyg primers. Line1 represents Hyper Ladder I, Lines 2-6 represent lines from family 7a, and Lines 7-11 represent lines from family 1a.

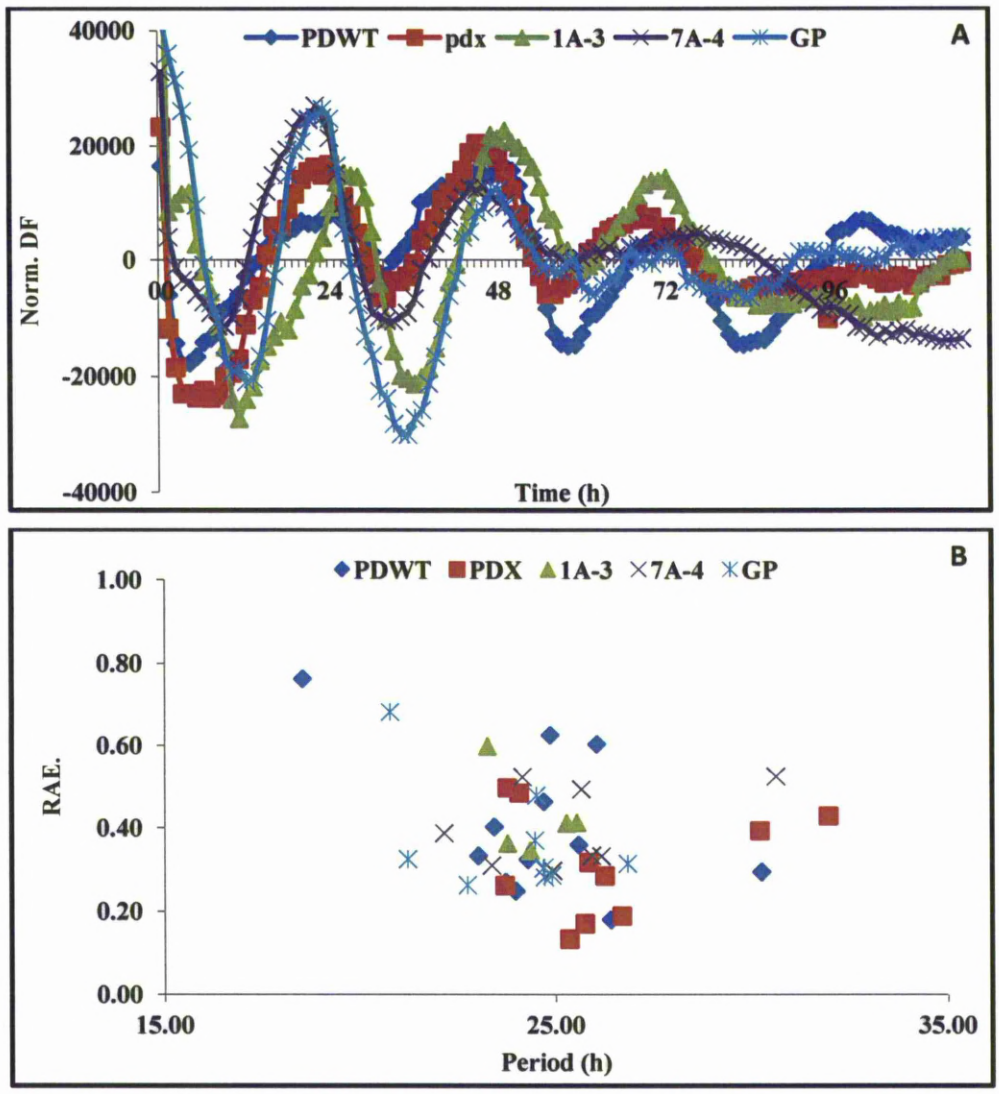

Figure 6.8. Comparison between DF rhythms of GP, PPd-H1 wild type and Ppd-H1 mutant with Ppd-H1-ox transgenic lines. (**A**) Normalized averages for DF rhythms of 12 leaf cuttings of four barley genotypes. (**B**) DF period plotted against their RAE estimated from leaves of four barley genotypes. The barley plants were entrained in long days (16:8 h light:dark) and high light over 500 µmol m-2 sec–1 for three weeks. The cut leaves were placed in constant darkness and exposed to 3 min of RB light 40 µmol m^{-2} sec^{-1} every one hour.

6.3. Discussion

Several repeatable and high throughput protocols have been developed to measure the clock function in *Arabidopsis*, including luciferase experiments, leaf movement, delayed fluorescence and measurement of transcript abundance of central clock components (Millar *et al.*, 1995; Gould *et al.*, 2009). However, these protocols have been adapted to suit the physiology and nature of the *Arabidopsis* plant and they are not applicable to measure robust rhythms of the circadian clock in all plant species. In this chapter, the delayed fluorescence protocol from *Arabidopsis* was adapted to measure bioluminescence rhythms in barley using barley leaf cuttings that were floated on water.

6.3.1 Optimization and measurement of DF in barley plants

In accordance with the results of this research, the level of DF rhythms was affected by gas exchanges (Figure 6.1). The DF emission from PSII likely reduced when O_2 was diminished to 1% O_2. That could be due to the higher energy demand of photorespiration as compared to CO_2 assimilation (Luttge, 2007). It was also illustrated that oscillation of DF rhythms was substantially influenced by the age of the barley plants used in the DF experiments (Figure 6.2). It proved difficult to distinguish the robustness of DF rhythms when using plants under the age of three weeks. At three weeks old, detection of robust DF rhythms is likely to be possible due to switching the photosynthesis or increasing of chlorophyll amount. There was twice as much chlorophyll in the mature plastids was twice as much as in compared to the developing plastids (Salomon *et al.*, 1987). However, these rhythms damped out after a couple of days or were unable to be detected by increasing the plant age. It is difficult to explain these results, but they might be related to the development of barley leaves during the growth stage.

The second major problem was that the appropriate light intensity was needed to maintain the vitality of the cut leaf until the end of experiments. The results of this investigation show that there is a strong positive relationship between the amount of DF signal and the light level used. Conversely, increasing light levels led to induction of early leaf senescence (Lim *et al.*, 2007). However, exposing cut leaves to the 40 μmol m^{-2} sec^{-1} constant light sustained their vitality for 5 days. Moreover, the DF oscillation pattern was greatly improved by entraining the barley plants under long day conditions with a high light of over 500 μmol m^{-2} sec^{-1} (Figure 6.5). The possible explanation for this might be that entraining the barley plants under long day conditions with a high light level led to stimulation of photosynthetic processes including an increase in PSII efficiency. Furthermore, running DF experiments in constant dark conditions provided a more reliable protocol for assaying circadian rhythms in barley plants. It helped to maintain a visualisation of circadian rhythms for a longer period of time.

6.3.2. Is the *Ppd-H1* gene orthologous to *PPR7*?

Circadian rhythms of both barley varieties *Ppd-H1* mutation and *PPd-H1* wild type were compared in order to characterise whether the point mutation in the *Ppd-H1* gene altered the circadian clock of the wild type *PPd-H1*. The DF results demonstrate that the *Ppd-H1* mutant line displayed robust rhythms that were similar to the *PPd-H1* wild type rhythms under constant dark conditions at 22°C (Figure 6.6, A and B). These results differ from previous research run by Cotter (2010), which indicated that both transcript abundance of the *Ppd-H1* mutant (run in constant light at 22°C) and DF rhythms (run in constant darkness at 17°C) had a period which was longer by 1 hour every cycle than the wild type. However, the findings of the current study support the previous research done by Cotter (2010) that *Ppd-H1* may be a key regulator in the temperature response in

barley. He found that transcript abundance of central clock genes of the *Ppd-H1* mutant displays a shorter period phenotype at 17°C than the wild type clock genes under constant light conditions, whereas at 22°C display a long period of transcript, and at 27°C show no effect or have a longer period than the wild type clock genes. Although the DF experiments were run under constant darkness, the DF data from this experiment illustrated that the *Ppd-H1* mutant displayed the same robust rhythms as the *PPd-H1* wild type at 22°C, while Cotter (2010) demonstrated that the *Ppd-H1* mutant displayed DF rhythms that peaked 1 hour after the *PPd-H1* wild type every cycle at 17°C. These differences of phenotypes displayed at different temperatures imply that the *HvPPDH1* gene might be involved in the temperature compensation mechanism in barley. In *Arabidopsis*, *AtPRR7* and *AtPRR9* genes are critical components of a temperature-sensitive circadian system and both genes play an important role in adjusting *CCA1/LHY* activities in response to ambient temperature (Salome and McClung, 2005; Salome *et al.*, 2010). As the *HvPPDH1*gene has been identified as most similar to the *AtPRR7* (Turner *et al.*, 2005), hence, the role that *AtPRR7* plays in temperature compensation may have been conserved in the barley PRR gene *HvPPDH1*.

PPDH1 has been identified as the major determinant of the barley photoperiod response and a member of the pseudo response regulator and FT-like gene families (Turner *et al.*, 2005; Faure *et al.*, 2007). Mutations in barley Ppd-h1 gene cause the late flowering phenotype and are most similar to the *Arabidopsis PRR7* mutation, which also causes the late flowering phenotype and has internal long period rhythms (Turner *et al.*, 2005). In order to confirm if the *HvPPDH1*is barley *PRR7*, DF experiments were run using ppd-h1-ox in Golden Promise samples, which were provided by Dr David Laurie. Although the ppd-h1-ox lines were still segregating, it is interesting to note that both ppd-

h1-ox lines displayed a period longer by approximately 2 hours than the wild type GP and shorter by 1 hour in comparison with the PPd-H1 wild type and mutant lines. Moreover, ppd-h1-ox line 1a-3 shows a phenotype of phase delay by 4 hours compared to GP. These data further support the suggestion by Cotter (2010) that *HvPPDH1* is not analogous to *AtPRR7* but to *AtPRR3*. Over-expression of *PRR3* in *Arabidopsis* also displays a phenotype of longer period and delayed phase (Murakami *et al.*, 2004). Moreover, transcript of *AtPRR3* peaks in the evening and was shown to be involved in the flowering pathway, which is similar to *HvPPDH1* (Para *et al.*, 2007). This finding also confirms that the ppd-h1 mutant is also a clock mutant. Further experiments to analyse the transcription of main clock genes using homozygous *PPDH1-ox* lines would confirm if this hypothesis is correct.

CHAPTER 7: GENERAL DISCUSSION AND FUTURE PROSPECTS

7.1. General discussion

The world's population increases every year; global warming, full use of areas suitable for agriculture, and severe shortages in fresh water all mean there is demand to create new crop varieties that are able to produce higher yields per unit area, are more stress tolerant, capable of irrigation with high salinity water, require less input, are able to cope with environmental changes (like high temperature), and can adapt to growth in more arid areas with fewer nutrients (Cotter, 2010; Rivandi, 2009). Previous work has revealed that endogenous circadian clocks that can be synchronised with changes in external time cues provide an adaptive advantage and increase the vegetative yield in *Arabidopsis* plants (Dodd *et al.,* 2005). *Arabidopsis* is a useful model for examining the circadian clock in plants. Therefore, the knowledge obtained from studying *Arabidopsis* circadian clock systems can be applied in order to identify and help to understand the importance of the clock in barley plants; and, ultimately, answer many questions, for instance: how can our understanding of the *Arabidopsis* clock system be applied to other crop species? Do the clock genes have similar functions and the same effects as their counterparts in *Arabidopsis* plants? How can the clock genes allow plants to respond to environmental changes and adapt their metabolism and physiological processes to cope with these changes? Consequently, improving our understanding of the circadian system in crop species may allow us to uncover mechanisms to further increase fitness and yields in crop species. Such data will provide plant breeders with valuable genetic resources that can be used to enhance crop fitness and increase yields in the future, or help to understand how and why specific circadian phenotypes are important to select for or

against, for example, it is not desirable to alter flowering time by altering the clock function in the whole plant.

7.1.1. Compromising the barley clock by knocking out of clock components

In order to discover what effects silencing clock genes had on the barley plant performance and to investigate similarity of the loss of function of of these genes in *Arabidopsis*, gene expression was knocked down using RNA-interference (RNAi). The reported data illustrated that losing the full functionality of *HvTOC1* and *HvGI* potentially had a negative effect on the development and survival of barley plants. In sharp contrast, the loss of *TOC1* function in *Arabidopsis* resulted in a short period and early flowering phenotype under short day conditions, while the loss of function of *GI* gave an extremely late flowering phenotype and decreased expression of *CCA1* under long day conditions (Mizoguchi *et al.*, 2002 and 2005). The current results confirmed that the fundamental clock genes of *Arabidopsis* circadian oscillators are likely to be conserved among monocotyledon plant species, however with slightly different roles. The present findings of this study are consistent with previous studies using *L. gibba* plants, which found that silencing *LgGI* using RNAi completely abolished the rhythmicity of two different circadian reporters (*AtCCA1:luc* and *AtTOC1:luc*) (Serikawa *et al.*, 2008). This suggests that, unlike *Arabidopsis*, *HvTOC1* and *HvGI* genes are essential for circadian oscillation and plant survival.

Future assays of silencing the evening genes and measuring clock transcripts at the embryo stage might provide a clear understanding of the effect of the clock on growth and development in barley plants. Furthermore, it would be interesting to investigate the

effects of over-expression of these genes in barley clock machinery and performance. Also, it will be important to develop a way of silencing clock genes in an inducible fashion.

7.1.2. Over-expression of barley *GIGANTEA* in *Arabidopsis* plants

To generate *Arabidopsis* plants that constitutively expressed *HvGI*, the *HvGI* gene was placed under the control of a *35S CaMv* promoter and transformed into *Arabidopsis* plants (WS and late flowering *gi-11* background) by using *PMDC32* vector. It is easy to discover the possible function of transformed *HvGI* gene in *Arabidopsis* plants from homozygous transformants that can be rapidly segregated, in comparison with barley transformation. These transformed *hvgi-ox Arabidopsis* plants illustrated a very similar leaf movement period to both WS and late flowering gi-11 mutants and exhibited robust DF rhythms that peaked earlier than wild type WS and *AtGI-11-ox.* Furthermore, the *hvgi-ox Arabidopsis* plants flowered earlier than wild type WS under LDs and completely rescued the late flowering phenotype of the *gi-11* mutant. This indicated that the function of *HvGI* had been extremely conserved through evolution, as this protein could interact with the *Arabidopsis* oscillator and flowering pathway to a similar outcome as *AtGI.* These results are consistent with several studies in *Brachypodium* to investigate the function of *BdGI* by over-expression of the *BdGI* gene in the *Arabidopsis gi-2* mutation which is fully rescued the late flowering phenotype of this mutation (Hong *et al.,* 2010); and in barley to examine the function of the *HvCCA1* gene by over-expressing it in WS background, which abolishes the circadian rhythmicity of delayed fluorescence and leaf movement in *Arabidopsis* plants (Cotter, 2010). This method, in fact, is very rapid and effective in detecting the function of the other clock genes in barley or in different plant species.

7.1.3. Quantification of the importance of light and temperature compensation of the circadian clock on the growth enhancement and fitness of barley plants

Environmental cues such as light and temperature can reset the clock mechanism and thus adjust the output rhythms with changes in daylight hours as the seasons of the year progress (Salome *et al.*, 2008). In this current study, variations of light and temperature had different implications on the circadian resonance of growth and fitness of barley plants.

The results illustrated that constant light strongly stimulated fresh and dry shoot weight, plant height, amount of chlorophyll content, RGR and ULR in all barley varieties tested except for LAR and SLA, which showed the highest values at T24. The highest values for LAR and SLA under T24 could be due to increase of leaf area and lower biomass and root shoot rations. Furthermore, the tested morphological parameters reached the maximum rates at 22°C, whereas they declined sharply on either side of this temperature. It is more likely that this enhancement of growth rates was influenced by the combination of temperature and constant light on chlorophyll per unit of leaf area. This effect may increase carbon fixation and photosynthesis per unit of leaf area, whilst the temperature might induce leaf area production rather than having an effect on photosynthesis. It seems that all genotypes widely adapted to the changes in both photoperiod and temperature. *PPd-H1* wild type seems to be giving the highest growth parameters across a broad range of environments, except for LAR and SLA. The most interesting finding is that the *Ppd-H1* mutant gave the highest value for leaf chlorophyll concentration, which might be a result of adaptation of the *Ppd-H1* mutant to constant light. This finding raised the possibility of the *Ppd-H1* mutant being a clock mutant and altering the biology of a sub-set of circadian regulated responses.

In general, conducting these experiments provided an insight into the impact of photoperiod and temperature on growth and adaptation of barley plants. In future, it would be interesting to run similar experiments with actual clock mutants such as loss of function or over-expression of barley oscillator genes. Data from such experiments would help researchers to understand whether accurate and robust clock function is an important feature in barley plants; and, moreover, how the circadian clock systems are adapted to cope with environmental changes such as photoperiod length and temperature variation.

7.1.4. Developing a circadian clock assay for barley plants using delayed fluorescence

During the last decades, a variety of protocols have been developed to rapidly and accurately assay the circadian clock in *Arabidopsis* plants including luciferase experiments, leaf movement, delayed fluorescence and measurement of transcript abundance of central clock components (Millar *et al.*, 1995; Gould *et al.*, 2009). Gould *et al.* (2009) showed that DF measurement could be used as a universal tool to measure the circadian rhythms in all photosynthetic organisms. This methodology had been originally designed for measurement of robust circadian rhythms in *Arabidopsis* plants and it was not applicable to all plant species. In this study, the delayed fluorescence protocol from *Arabidopsis* was modified to measure DF rhythms in barley using leaf cuttings that were floated on water in order to overcome the issue of plant size.

In accordance with the results of this study, the robustness of DF rhythms was affected by gas exchanges, leaf age, intensity of light and temperature. Initially, the DF rhythms could only be detected by placing the barley leaves in constant RB light (40

μmol m^{-2} sec^{-1}) at 22°C after entraining the barley plants in long days at high intensity light (over 500 μmol m^{-2} sec^{-1}) for three weeks. To create a practical and reliable protocol for analysis of the circadian rhythms in barley plants, DF experiments should be run under conditions of constant darkness (only switching the light on for 3 mins before taking a 1 min exposure) in order to maintain a visualisation of circadian rhythms for a longer period of time (up to 96 hours); this protocol gave the best and most reliable results.

Furthermore, DF rhythms were used to investigate whether the *Ppd-H1* is a barley *PPR7* gene and to ensure that the point mutation in the *Ppd-H1* gene altered the circadian clock of the wild type *PPd-H1*. The DF oscillation pattern of *PPd-H1* wild type and *Ppd-H1* mutant were compared with transgenic lines of *PPd-H1* over-expressed in Golden Promise. In spite of the fact that the *ppd-h1-ox* lines are still segregating, the observations from this study indicated that *HvPPDH1* is not analogous to *AtPRR7* but to *AtPRR3*. Some of the ppd-h1-ox lines exhibited a phenotype of phase delay and long period in comparison with wild type GP and a period shorter by 1 hour than PPd-H1 wild type and mutant lines. Over-expression of *PPR3* in *Arabidopsis* also displayed a phenotype of longer period and delayed phase (Murakami *et al.*, 2004). Furthermore, transcripts of *AtPRR3* peaked in the evening and are suggested to be involved in the flowering pathway, which is similar to *HvPPDH1* (Para *et al.*, 2007). This finding also confirmed that the *ppd-h1* mutant is a clock mutant with a period longer by 0.5 to 1 hour than wild type at 22°C. The late flowering phenotype of the *ppd-h1* mutant is more likely to result from a lack of *HvFT* transcript induction due to high degradation of *HvC02* protein in the dark as a result of the long period clock phenotype of the *ppd-H1* plants (Cotter, 2010).

There are a great number of barley varieties adapted to different climate conditions or specific environments (abiotic stress tolerance) over the globe. It is possible that some of the existing barley mutants could also be clock mutants. In the future, it would be interesting to use DF rhythm assays to screen the existing mutant collections for clock mutation.

7.2. Future prospects

This study provided an overview of barley central clock genes and supports the hypothesis that the circadian clock is conserved between monocotyledonous and dicotyledonous plants. However, the precise functions of the clock gene in the circadian clock system appear to differ. Further work is required in order to achieve a better understanding of how the circadian clock machinery operates in barley.

1- Silencing the barley evening genes (*TOC1* and *GI*) had a major effect on the development and survival of barley plants. In the future, it would be interesting to investigate if the silencing of the morning genes (*CCA1*) had a similar effect on the growth and development of barley plants. Moreover, it would be interesting to understand the effects of these genes on the barley circadian oscillator and input and output pathways through measuring the main clock gene transcription at the transformed embryo stage.

2- Over-expression of the *HvGI* gene in the *Arabidopsis* late flowering mutant gi-11 background completely rescued the late flowering phenotype under LDs, and this suggested that the *HvGI* gene is an *Arabidopsis* GI gene orthologue, functioning as a regulator in both the circadian clock oscillate and photoperiodic pathway. It would

also be interesting to measure the transcription of both clock components and flowering pathway genes in these lines.

3- *GI* is suggested to play a critical role in the temperature compensation of the *Arabidopsis* clock, by extending the temperature range over which robust and accurate rhythmicity can be maintained (Gould *et al.*, 2006). Therefore, it would be interesting to investigate whether the *HvGI* gene is a barley key regulator at a temperature level similar to its counterpart, *AtGI.* One way to approach the issue would be via conducting leaf movement and DF experiments under different ranges of temperatures using the *35S:HvGI* transgenic in WS and late flowering mutant gi-11 background.

4- It would be interesting in the future to assess the effects of *HvGI–ox* on the circadian clock and flowering time in barley plants.

5- The primary results of DF rhythms illustrated that *HvPPDH1* is not analogous to *AtPRR7* but to *AtPRR3*. To confirm this hypothesis, a DF experiment similar to this one should be carried out using the homozygous *PPDH1-ox* lines after they are produced.

6- In *Arabidopsis* plants, the *PRR3* gene functions in stabilisation of the *TOC1* protein in blue light by competing with *ZTL* to bind to the *TOC1* (Para *et al.*, 2007). Future experiments measuring the *TOC1* expression in homozygous *PPDH1-ox* lines would help to establish a greater degree of accuracy regarding this matter.

7- It would be practical to develop accurate and reliable protocols suitable for measuring robust rhythms in crop species in the future, such as measuring stomata

conductance, or manipulating luciferase marker genes by fusing them to the clock-related genes in order to follow the circadian rhythms at the embryo stage (callus material). Furthermore, manipulation of the existing technologies of analysing gene expression and characterizing their related biochemical function (such as microarray) would create powerful tools to study the basis of the circadian clock system in different plant species.

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APPENDIX 1: Sequences of Barley Genes used in the study

Sequence of barley genomic *PRR1 (TOC1)* gene: positions of the primers that have been used to amplify target DNA are illustrated by shadowing (forward primer in yellow and reverse primer in blue).

TTGACCGGAGCAAGGTGAGGATCCTCCTCTGTGACAGCGACCCCGACAGCTC
ACAGGATGTGCTTCGCCTCCTCTGTAACTGCTCCTACCAAGGTCAGTTCACCT
GAGGCTTGTTAACCATGTGTTTCATGCTTGCTTGTTAATCATGACGAGTATTA
TTATTACCGATGCAACTAGTATCACCATGTTCTCTGAATCTCTTCTATTGACGT
ATCTGGTGCTCGGTGGTGGTGGTATTGCTACCTTCTTTTTCAGTGACCTGCGC
CAAGTCTCCACGGCAGGTGATCAACGTGCTCAACTGCGAGGGCGCTGAGATA
GACATCATCCTCGCCGAGGTCGACCTGCCAGTCTCCAAGTGCTTCAAGATGCT
CAAGTACATTGGCAGGAACAAGGAGCTGCGTCACATCCCCATCATCAGTAAG
CTGTCTTCCAGTTCCCATAATTCTGTCCAATTCCTGTGCCATGCTTGTATTGAT
GATATTCGTACGGATGCAATTACGCAGTGATGTCCAATAGAGACGAGGTTTC
TGTTGTTGTCAAGTGCTTGCGGCTCGGGGCAGCCGAGTACCTGGTCAAGCCG
CTTCGCATGAATGAGCTCTTGAATCTGTGGACCCATGTGTGGCGAAGGAGAC
GGATGGTAAATGGCAATTTGTTTTTTCACCTTCGCTGGTAGTGTTAAATAAAA
GAATGCTCTAAGCTGGTATGCATGTGGTCGTTGCTCTACTGTTTATGAATTGT
TCTTGAACTGAATATTGCTGTTGGCAGCTTGGTTTGGCCGAGAAAAACTTCTT
CATTGACAATCTTGAGTTGGTGCTATCCGAACCTAGTGATGCCAATACCAATA
GCACCACACTCCTTTCGGATGAGACAGATGACAAGCCAAAAGGAAATAGAA
ATCATGAAACAAATACCTCGAGTCAATATGAATATGAGGTAATCAACTAATT
TATCTGTAGGATGTTCTACAACTGTCCTATGATTGGTTTTCTCTTCCTTTCTCT
TTAGTATCTTCCAAAATTTATGTTGCCGCAAGTATGTGCTTTTTTTTTACCATA
TATGCTGTACAATGATGAGATGCTCATTGCTGTGCATTAAGTTACTTATTAAA
CCCTTACTTTTCTTATCATCCCCAGTCTCCTGCTACGGACCCCCCAAAAACAG
ACCAATTGGAAAATTTACCTAGCATTGCGGAAGATGATGACAATGCATGTAA
GTGTCTTCAGTCTAACCTTTGATTTCCTCTTCAGCTGTTTTGTTTTTGTCCCCTC
TCATACTACCTACTCATTCATAGTTCTCAGCTGGTTTCTAAAGCTTATCTTTTT
ACCAGCATCTCCAGGAGGAATGTTTTCACGCCCAATAAAGACTAATTTGAGG
ATAGCTGAGTCATCTGCATTTCTAGCATATGTTAAATCAAGCACTCCAGCAAA
CAGCTCATTGGATACTGAACTACAGAGAGGGGCTAATAAGTTAGATATTGTG

GATCACCAGGTTAATTTCTCCGGTTCGACTGACAGAATCGACACTAATAGCA
GTACAAATATTCAGGATGAAAAAGCTTTTGAGATGCCCACGCAGTATCCTTT
GGTTTGCTTTTCTTCCTCTAACTTGCAACTGGAGCAAAGGAATGAGGGTCAAC
AAGATGTTTCAGGAAACCCTCCTGTATATCATTACCCATTTTATTATCCAGGG
ATGGTAGAGCATAGCATGGCACTTCATTCAGTTCAAAGTTTCCAAGGAAACA
TAAACACTGCTCAAGCTCATACACCACCAACAATGCTCCATCAATACAGTGTT
TATCACCAATCCCATGGTGCGTCTACAATGCCATCGTATCAGTACAATCCTGC
TGGTATGAATGTGCATTCAAGTCACTTGTCAATGCAGAATGTGTGGTCATCGG
TATCGAGCACACCAATTTCCGAGGAAAGACATGGTCACTCTGGCAGGAGGGC
TGCAGCACTTGCAAAATTCAGGCAGAAAAGGAAGGACCGCTGTTTTGACAAG
AAGGTGAGGTATGTTAATAGGAAGAAACTTGCTGAAACAAGGCCGAGGGTG
CGGGGTCAGT

Sequence of barley genomic *GI* gene: positions of the primers that have been used to amplify target DNA are illustrated by shadowing (forward primer in yellow and reverse primer in blue

CATTTTAATTCACAGCTAAACTCAAGGCCAAAAGCAGAAATCTCGAAAAGAA
GCGGGGTGTTGTGTGTCTGAGAAATTTTACTTGCTTTTGAAGATATATCTTGA
TGTGCAGAATTAAACTCCTTTTGGTTCTGCTCCTGTTCAACTTCCGAGGGTTA
CGTGTTGGAATGGATCTATATTGCATTTGTCTACTTGCTATATTTAGCGCGTG
GATGGATCTAATCGGATTTTCTAAATTAGAAACGTGCGATGTTCGATAATAG
ACAAAAACATGGTAAGGCAAGTGAACTACCAATATTGTTTTACCCAAATTTG
GTTAGCAGTCATCTGACTTGCAGTTATTGCCCCTCTGAAATATGTGGATTGTG
TATTATTATGTTCAGATTTGTATGAACTTGTACAGTAGTTATAAGCTTACAAC
GTAGCTCTGCTATTGTTTTTTCTTTCTCCTTTTTCTTCATTCTGGTTTCTGTTTCT
GGGGTCCCAGTTCCGAGTTGTTTTCTTTGTTTCTGCGTTGTGCCATTTTGGTGT
TACTCATCTAATACAAACGGCACATCTTTTGGTCTGATGTGTCATGTGTGTTC
GAGAAAGACGGCATATATCTTTTATTTCTTTATTGAGAATGGTTGCTCCTTTTC
TAGATGAGAATTTGATGCTTCTAGTTGTGTAAAAATACATCAATTTTACACAC
ATAAGCTCTTTTTAAAGTCCAAAAGACAATCCTCAGCTCCGAAACAAAACAG
TTCAATTTGCAACCTAAAACGCCCCCCCCCCGCGCGCGCGCCAAAAGAAA
GAACATCTTTTGGGAGACGTGCCATTTACGTCCAAGGCAGCCATCTCTCCAAC
TCGAGGAATCATGTTGAGCAGAATTGGGTCTGGGACCAATGATGAAGATGAG

TAGATGACTGAGCCCTTGGATCCTGATCCAAGGGTCAGAGTTGGCGATTAGA
TGTGTTCCTATACTATCTTATTTCCCCAAGCATCTGGAATCATTCCATTCCATC
CCATTCTCAAATCTCAAAGTCCAGGATGGTTCCATTCCAGTCTACTTCATTCC
TCAAACCACTAACGGTTTTAGAGTTGAAATCCTTTTTCTGACTCTGTTAAGGG
CTGGGAATGTAAACGAGACTTCTTTTTAGAAATATGTTGCCAAGGCTA
GCAAAATTTGTTCCATTGGCTGGTTGACCTCCTCTCTTGTCAATTTATGGCAAT
ATTTCTTGTTTTAAAATTTATTAAAATGACATCTATAATAATGTCCCTTTCCTT
CTTTCGACTTTCCTTTTGCTATATTTTGCAATGCAGAGACGTTAAGCGCCTATG
GTTGGAACTGGTTGATATGATATCTGGTCACATTGTGGGATGCAATCCCCCCT
TAGTGGGGGGTGATTGAAGAAGTCAACAATAGGAGAACTTGCATCTTGCTGA
AGTTTTCTGGTACTGAGGCAAGTAGCTTATAAGAAGAATAGGGAATGGCATA
GATGGATAACTGACCCGCTATTTTAGCATGCTGTTGAATATGCTTTTATTGCT
AACTGGGGCAGTTTTTCTAATTTCTTTCTTTCTGAGTGTTCCTCTCTAGTTTCTT
GAGCAGGCTCTTAAACTACCAAGTATGTCAGCGTCAAATGGGAAGTGGATTG
ATGGACTCCAGTTCTCATCACTGTTCTGGCCCCCGCCACACGATGCGCAGCAG
AAACAGGTACAATGCTCATAGTTTAGCTATGTCATATACGCTATAACTTTGTA
AACACCTGATGTTAATGCGACACTTATGTTTTCTTTTCTTTTTGAAGGCACAA
ATTTTAGCCTATGTTGAGTACTTTGGTCAGTTCACATCTGACAGCGAGCAATT
CCCGGAGGATGTAGCTCAGGTACTGTATCTCTTGGCATAAATTAGTTTTTTTT
GTGGTAAAATTTATATCAACTGTTAATATTTAGACCCCTCCATCTTTCTTGCTT
GTGTGCAGCTAATTCAAACTTGCTATCCATCAAAAGAAAAGCGGTTGGTAGA
TGAAGTATTAGGTAACATATGAACTTCTGATAGCTACACATTATTACTACATG
CATATGAAGAAAGAAATTGACACTCTTGTTTTTGCATTTCGCCCAGCAACTTT
TGTTCTCCATCACCCCGAGCATGGTCATGCAGTTGTACATCCAATTCTTTCAC
GCATCATAGACGGGACACTGAGTTATGATAGTCATGGTTCCCCATTCAATTCC
TTCATCTCCTTATTTACCCAAAGTTCTGAGGTGAGGCCCTTACTTCCCTGGTTA
CTTTCAGTTCTCTTGCTTGATGCTGGTTGTGGTTGAAGTTCTGTTGCTCATGAA
GTGTACTTTTGGCTGCAGAAAGAGTACTCAGAGCAGTGGGCCTTGGCCTGTG
GAGAGATTCTTAGAGTTCTAACTCACTACAACAGGCCAATCTTTAAAGTTGCA
GACTGTAACAACACCTCTGACCAGGCTACAACAAGTTGTTCTGCACAGGAGA
AAGCTAATTACTCTCCAGGAAATGAACCTGAACGGAAGCCATTGAGGCCATT
ATCTCCTTGGATCACAGATATTTTGCTAACTGCACCTTTAGGCATTAGAAGCG
ACTATTTTAGATGGTGAGTTGACTTTAATTTCTTTTTATTTTTTCTTATGAAAC
ACAACTTAAGGTAATGTGACTATTAATAAGGAGCAAAATTAGCGAATGGAGA

AACAGGGTAAATAGTAGAACCACGCGAGGAAAATAGTATTGCAGGTGAGCA
ATAAGGCTGAAACTCAGGAGTAAGTGGCCTGTAATGAACACCACACTGCAAG
CATCCCCGGTATGCTAATGGGAGTTTGTGCTAATCAACTCCAAATATGCTAAT
GGCAGTGTGCCAATCAACTCTAAATATACTAATGGGAGTGTATGCTGTACAT
GAATAGTAAATGTCAACGCTTTTCAGCTTGTTGGAATATATGCTGTTACTGTT
GTAAATCAACAGTTTGCTATCATACACTTCGCTTAGTAATATTAGCTGATTAC
TCTCTGTTTCATTTTAAACCTATCAGGTGTGGTGGAGTTATGGGAAAATACGC
AGCTGGTGGAGAATTGAAGCCTCCAACAACTGGTTAGTAATTTTGAAATCAA
CTTTTGTGCCTGAAATGAGAAGATGCAGTAACACTAGATGTTTTCTTTGTTCC
ATCGAGATTTTATGTCTTTGTTGCTTCTTATTTTTATTATGCGGGTTTTCCAAG
TGTGGAACACCTTTTTTTCTTGCAACAGATGCTAATTCTTGCCATAAACAAAA
ATGATTAATTATACGTCCTTTTAATTTAGCTTACAGCCGAGGAGCTGGTAAGC
ACCCACAACTCATGCCATCCACCCCAAGATGGGCTGTTGCCAATGGAGCTGG
AGTTATTTTAAGTGTCTGTGATGAGGAAGTAGCTCGTTATGAGACAGCAAAC
TTAACCGCGGCAGCTGTTCCCGCCCTTCTGCTACCTCCACCGACAACACCCTT
GGATGAGCATTTGGTGGCAGGGCTACCCCCTCTTGAGCCATACGCTCGCTTGT
TTCATAGGTAAGATCCTATCATTTGTCATACCAAGTCTGCTTTCCGGATTGTA
CTAAACGTGTTACTCTCTTGTCAGATACTATGCAATTGCCACACCAAGTGCTA
CACAAAGATTGCTTTTTGGTCTTCTTGAAGCACCACCATCATGGGCTCCAGAT
GCACTTGATGCAGCAGTTCAGCTTGTTGAACTCCTTCGGGCAGCTGAAGATTA
TGCTACTGGCATGCGGGTATGACATACTGCATGCTGGCTGTTGTTTCAGTCCT
GTTAGTTGTGATGCCTCACGATACAAAATTTCCATATTCGTATGTTTTGGGTG
TGCATGTTTACTAATCTTGGTAACTTTAAATTCCTGTTCAGCTTCCAAAAAATT
GGTTGCATCTTCATTTCTTGCGTGCGATTGGAACTGCAATGTCTATGAGGGCT
GGTATTGCTGCCGATACAGCTGCTGCGTTGCTTTTTCGCATACTATCCCAACC
AACGTTGCTTTTTCCTCCACTAAGGCATGCTGAAGGAGTTGAAGTGCAACATG
AACCACTGGGTGGCTATGTATCATCATACAAAAGACAGGTATGCAGTAGTTT
CTGCATCTAGTTAATTTTTCATTATCTGTTCTTCTTTAGTAAAGACTCAAAGCA
TAGTTAGTCTTTTCTGGGGCTTATGTATTCTAGGCTCTGTTGTTAAGTTCAGGG
GATCTTATTATACACTGATTTAGGCATTGTTAGTCACAGTTTACGTTACTACT
GTTAGGAAAGCAACTTGTCTATATTGAAGGCCCAAGGGGCATATATATATTA
CACATGACTTGAGGTGCAAGGAAAGTAAAGATAGACTAATAAGGACTCCTAG
ACTAATACTAATAGACTAATAAGACTCCTAGACTAATACTAATACTTCCTAAC
AACTACCTTAGTTGAGCTAAAACACTGCAACATACAGATAATCCTTATAGTTA

ATTTCCATTCAACATTTGATTATTTTTGCATACTTGCTCATGTGGAATGCTTCT
ATCAATTCCTCAATCACGTATCTTGTGATTTACCATGTTCTATGACTACTGTTT
TAGAGATTTCACACCAAAGCTGCGCATTCAACTATCGTCTTTATTTACACCCT
TCCATAGGGGAATCCATGTTTTAGTCTTCTTGGTTTTACTGATTGTTGCCTTAT
GTCTGCATGACTAATTTACCTGCTTGCACTTTGAACTATTCACAGCTGGAAGT
TCCTGCATCTGAAACCACAATTGATGCCACTGCACAAGGCATTGCTTCCTTGC
TGTGTGCTCATGGTCCTGATGTTGAGTGGAGAATATGTACCATCTGGGAAGCT
GCCTATGGTTTGTTACCTCTGAATTCATCAGCAGTTGATTTGCCCGAAATCGT
TGTAGCTGCTCCGCTTCAGCCACCTACTTTGTCATGGAGCCTATACTTGCCAC
TGTTGAAAGTATTCGAGTATCTACCTCGTGGAAGTCCATCTGAAGCATGCCTT
ATGAGAATATTTGTGGCAACAGTTGAAGCTATACTCAGAAGAACTTTCCCTTC
GGAAACCTCTGAATCATCTAAAAGACCAAGAAGTCAATCCAAGAACCTTGCT
GTTGCTGAACTCCGTACAATGATACATTCACTCTTTGTTGAATCATGTGCTTC
AATGAACCTTGCTTCCCGGTTGTTGTTTGTTGTATTAACTGTTTGCGTCAGTCA
TCAAGCTTTGCCAGGGGGCAGCAAAAGACCAACGGGTAGTGAAAACCATTCT
TCTGAGGAGGCCACTGAGGACCCAAGATTAACCAATGGAAGAAATAAGGTC
AAGAAGAAACAAGGGCCTGTTGGTACATTTGACTCGTATGTGCTGGCTGCTG
TTTGTGCCTTATCTTGTGAGCTTCAGCTGTTCCCTATCCTTTGCAAGAGTGCAA
CAAACTCAAAAGTAAAAGACTCTATAAAGATCCTGAAGCCTGGAAAAAACA
ATGGGATCAGTAATGAGCTACAGAATAGCATTAGCTCAGCAATTCTCCATAC
TCGTAGAATTCTTGGCATCCTGGAAGCTCTTTTCTCCTTGAAGCCATCATCAG
TTGGTACCTCCTGGAACTATAGTTCAAATGAGATAGTTGCAGCGGCTATGGTT
GCCGCTCATGTTTCTGAGTTATTTCGCCGGTCGAGGCCATGCCTAAATGCACT
ATCTTCACTGAAGCGATGTAAGTGGGATGCTGAGATTTCTACCAGGGCATCA
TCCCTTTACCATTTGATCGATTTGCATGGTAAAACTGTGTCCTCCATCGTGAA
CAAAGCTGAGCCTCTAGAAGCTCACCTGACTTTTACATCAGTAAAGAGAGAT
GGTCAACAACACATTGAGGAAAACAGCACCAGCTCATCGGGTAATGGCAACT
TGGAAAAGAAGAATGCTTCAGCCTCACACATGAAAAATGGTTTTTCAAGACC
ACTCTTGAAATGCTCAGAAGAGGCTAGGCGAAATGGTAATGTTGCAAGTACA
TCCGGGAAAGTTCCTGCAACTTTACAGGCTGAAGCATCTGATTTGGCTAACTT
CCTTACCATGGATAGAAATGGGGGTTATCGAGGCTCTCAGACTCTCCTAAGTT
CTGTTATCTCAGAAAAACAGGAATTATGCTTCTCTGTTGTCTCATTGCTCTGG
CATAAGCTTATTGCATCTCCTGAAACGCAGATGTCTGCAGAAAGTACATCAG
CTCATCAAGGTTGGAGAAAGGTATGATTCGTGTGGCCATTGGCATAACACAG

CAATGCTTCAGTGACATTCTAATTTTGTTATCTTATTAACTGTACATCTGACAT
TTTTTACGTATAGGTTGTAGATGCCCTTTGTGATGTTGTTTCAGCCTCACCAGC
CAAGGCTTCAACTGCTATTGTTCTGCAGGTAAAGATTTATTCAGAGATTTGGC
CGCACTGAAATTTGGTTCAGATTTTGGCTTCTTGTCCCTTCATTTCTCATGTGG
GAACACACATTGTCTTCATGACATATACATATGTATTTCCTGTCATATTTATG
GACTCTTGTATGCAGGCTGAGAAGGACCTACAGCCCTGGATTGCTCGAGATG
ACGAGGAAGGTCAAAAGATGTGGAGAGTCAACCAGCGAATAGTTAAACTGA
TAGCTGAGCTTATGAGGAACCATGATAGCCCAGAAGCATTGATAATTCTTGC
TAGTGCTTCAGACCTTCTGCTTCGTGCTACAGATGGGATGCTTGTTGATGGTG
AAGCTTGTACCTTGCCTCAGTTAGAGGTAAATACATATACAAAAGTTCCTTCA
CAGAATAGCACAAACATATCATCTGTTGTACTTTATTGTCGTTAAAGCAGCAT
CGACTCATTGTCTTGCTTTGAAGTTGCATATCATCGATTCATTCTGACAACGTT
TGTTCCTGTGATTGAATACGCACAGCTCCTGGAAGTAACTGCTAGAGCTATTC
ATCTCATCGTTGAATGGGGAGACCCAGGTGTAGCAGTTGCTGATGGCCTCTC
AAATCTGTTAAAGGTATGTCTTTCGTTCAGATCTTACCCCTAATCTGTTCATCA
GAAACACAACCACGTTTAAAATGCTAATGAGTCAATGTGGTTTGCAGTGCCG
TCTATCGCCTACCATCCGCTGCCTCTCCCACGCTAGCGCACACGTACGAGCGC
TCAGCATGTCCGTCCTCCGTGACATCTTGAACAGTGGACCACTAGGTTCCAGT
AAGACCATTCAAGGCGAGCAACGGAACGGCATCCAAAGCCCAAACTACCAA
TGCGCGGCAGCAAACACGGTGAACTGGCAAGCGGATGTCGAGAGATGTATA
GACTGGGAAGCCCGCAGCCGCCGTGCCACCGGGATGACCCTCGCCTTCCTCA
CCGCTGCTGCTAACGAGCTGGGCTGCCCCCTTCCTTGCTGACAAGGCCATATT
TGAAGCTGACAATCAGCAACACTTGACAGTTGGTGCGAGCAGTTGCTGCATG
GTCAGCGAGCAGGATGGCTAATTAATCCCTTGCTCAAGGATGGCTTTCCATTC
CCAGTCTGCCCCCCATGATGTGATGTATATTAGCTGATGTTCCCAATCATACG
GAGCTTTGCTCCCGCGGTGTGATTTTAACTTGCAATCTGACGTTAGATGTTCA
AGCATATTGAACTGCTTGTGCTGA

Sequence of barley genomic *CCA1* gene: positions of the primers that have been used to amplify target DNA are illustrated by shadowing (forward primer in yellow and reverse primer in blue).

CGCGTCAGGTCCAGCGTTTGGCTGGAGACACAGAAGGAGGAGTGCTGTTGTT
TGTAGACGCCCTCAACTCCAAGTAACAAAGCGACCAGTTGTCAGCACTTCCTT
TTGTTTTCGCATTTCCCTGGAATTGGAGATGGAGATAAATTCTTCGGGTGAGG
AAACGGTGATAAAGGTGAGTTGCTCTTGGACCTACCAGTTGTTTTGTGGTGAA
AGAATCAACATTTTCTTCATGTTGTTTGTAGGTGCGAAAGCCGTACACAATAA
CAAAACAGCGGGAGCGGTGGACTGAGGCAGAGCACAAACGGTTCCTTGAAG
CCCTCAAACTGTATGGCAGAGCTTGGCAGCGCATAGAAGGTGAAATCTTCAT
ATATGTCATACCTTGAGTACTTGTACTCACTGATTTCATCTAAGGAAATACTC
ACTGATAAAGGTGTTGCTTAATTCATCAATTTGCACTCACTGATTTCTTCCAA
GGAAATACTCACTGATTATTAGTGGTATATAATATATGTTAATTACTTTATTA
TTGGTAACTTTATACAAACTCATTTATTTTATTTTCTTATTCACCTTCTGGTTTG
TTGCACAGAGCATGTTGGGACAAAGACGGCCGTGCAAATCAGAAGCCATGCT
CAGAAGTTCTTCACAAAGGTTATTTTTTCTATCTTATTGGCTAGTACCCTTGTT
TGGTTTACTGCTATACCCTTCGTTTTCGGGCTGTTTCATCTTTATTGTTAGTGA
TAAAATGAAGACTTGCGCATGCTTCATAACTTTTATGTCTTTATTTTACTGAA
GTATTTTCATTTTTTCTTTCTTTGTACAGTTTGTTCTGCAGTGCTGAGCATGAT
GAAAGATTCGTTTCCTTTGAATATCATGAGAACCATGCTTTATCTAATTGTTG
CCCTGCTGATTGATTAAAAGATCAAATTAAAGCATGTTAAAATGGATGAAAC
ATGCTAGAAAGTCTTTGCTGATTGTAAGCAATGAGTAATGTTGCAGAGAAGA
TGATGTTGAACAACTCTGATTCTCTCATTGATTCATTTTACATATCCATGCTGT
TATTGAACATACATATGACTAAGAAAACATTATGGTACTAGTTAAATTTACAT
AGAGAGTCAATATAATACATAATGTGAAATAGGATATCTGAATGCACTCAAG
TGAACTACAGGAAATTCCTTAGGAATTGTATGGTAAATATGCTAACATTTGTT
GCATTTCATTATAGCCCCCATCAACGTAGGTAAACACTTTATCTAACATGTTT
CACTATCAACATTGTGTAAATGAGTCCATTTCATTTTCTAAATCATGATATTTC
TTCCGCAGAAGTACACAATGGCATGAACAAAATTTATCTTCTTTCTCCCCTTT
TTGTCCTTGCTCTCTTCTCTGCTTTCTGTTCTTTGCTGCTTGTCCTATTTTTTTA
GGGGCTGCATACAGCCACATGGTGGAGGCTACATTGCCAATTTGCCATCATG
TTGTAACATGCAACAGACTGTTGCCTTGTTAGGATTGCAGCCTCATCCTCTCA
GAGGTTTCTTTGCCAGACCCATGGAGGAGGGAGGAGGCTAAGGCTAGTCTTT

TCTTCCCTTGTTTTGACCACGATGGTTTCTGGGTCCTCAAATTTCATACTTTGC
TCACCAGCCATTACCAATCTGACCTTGCTCAATATAGCAGGTTGGTTGTTGGG
ATGGACAGCTCTACCCTATTGTACATATACATATATATATAGTAAATGACGCA
ACTAACGGTTCAGTACACCAACTTAGCTCATAGTTACTCGCCAATAAGTTTGT
AAAAGCTCAGGGTGGAAACTGTCAGCCTTCAAGTTCAATCTAACCAATAGTT
TAAAGGTTTTGTGCATCGGTTGATGGAGAGGCTGGAGAGATGTCTCTTCCATA
ATCCAAAAACAATTTTACAGTGCTATTTTCTTACCTTTTATGTGGAATAGAGA
AGAGCCAGTAGAGTGAAAAAGAAACCACCATCCACTCTTTCAGTCCTCCACC
CGCTTGAGGGTTTAGGCTGTAGAATGCTGTATTTGTCAGGGTTAGGATACCGG
AACTGCCCCAAATTCCCATTGTAGGCGGTTCTGGTTTCTTGCAGAACCTGGCC
AGTTAGTCTGGTTGCCCAAAAATGCCTTATTTTCTGGTGCTCTGAATCTTCATT
TTCATTTTGCAGATTTCAAATTGAAGATATAAATTACAGACCTATGCAACAAT
AGATAGTTCAATTTTCCAATTCTAGTTTATCACCACTGTCTGTCTGTATCTAAC
TATTTGGGAAATTGCAGTTGGAAAAGGAAGCTATCAACAATGGTACTTCTCC
AGGACAAGCTCATGATATAGACATACCTCCACCACGGCCTAAAAGAAAACCT
AACTGTCCATATCCTCGAAAAGGTTGTCTCAGCTCTGAGACACCCACCAGAG
AAGTTCCAAAATCAAGTGTTAGCTTGAGCAATAGCAATTCACAAATGGAAAG
CAATGGAACTCTTCAGGTAAATACCGTTGTAGTATTATATGCTTTGATTTTAG
TTCTATCTATTCTATCTATTCTATCCTTGATGTATATATTGATATACTTGTTTTT
CTTTAACATGAAGGTCACCAGCACTCAGAAACTTCAAAGGAAGGAGTTGTCT
GGAAACGGCAGTTGCTCAGAAGTTATTAATATCTTTAGAGAAGCACCATCTG
CCTCATTTTCTTCCTCTAACAAGAGCTCTTCAAATCATGGTGTCTCTGGGGGA
ATTGAACCGACTAAAACAGAAATCAAAGATATGGCAGCCATGGAAAGGAAA
TCTACTTCCGTTGATGTGGCGAAGGATGTAAAAGATATTAATGACCAGGAAA
TGGAAAGGAACAACAGAGTCCACATCAGTTCTAAATATGACCGTTCTCATGA
AGATTGTTTGGATAGCTCAATGAAACACATGCAGTTGAAGCCAAATACTGTG
GAGACAACATACACGGGTCAACATGTTGCAGGTGCTCCACTCTACCAAATGA
ATAAGACTGGGGCAACTGGCACTCCAGACCCTGGAACTGAAGGAAGTCATCC
TGATCAAACGAATGATCAAGTGGGAGGAGCTAATGGAAGTATGGACTGCATC
CATCCAACACTTCCCGTGGATCTAAAATTTGGCAGCAGCTCCACAGCGCAGC
CCTTTCCCCACAACTATTCAGGCTTTGCACCAACGATGCAATGCCAGTGCAAC
CAAGATGCCTACAGGTCATCTGTTGATATGTCGTCCACCTTCTCCAACATGCT
CGTTTCCACATTGTTATCAAACCCCACAGTACATGCAGCTGCAAGGCTTGCAG
CATCATACTGGCCAGCAGCAGACAGCAACATCCCTGTCGATCCAAATCAAGG

AATTTTTGCTCAGAATGCTCAAGGAAGACATATTGTTTCTCCTCCAAGCATGG
CTTCTGTGGTAGCAGCTACAGTTGCTGCGGCTTCGGCATGGTGGGCAACACA
AGGTCTTCTCCCTCTTTTTGCTCCCCCCATGGCTTTTCCATTTGTCCCAGTTCCT
ACCGCTTCCTTTCCCACAGCGGATGTCCAGCGAGCTACAGAGAACTGCCCAG
TGGACAACGCACCAAAGGAATGCCAAGTAGCTCAGGGGCAAGGTCAACCTG
AAGCTATGATAGTTGTAGCATCTTCTGGGTCCGGCGAGAGTGGAAAAGGAGA
GGTGTGTCCTCACACTGAGTTAAATATATCTCTGGCTGATAAAGCTGAGACA
ACACCTGCCACAGGAGCTGAAACAAGTGATGCTTTGGGCAACAAGAAGAAG
CAGGATCGCTCTTCATGTGGTTCCAACACACCATCAAGTAGTGATGTAGAGG
CAGAACATGTTCCTGAGAACCAAGATCAAGCTAACGACAAGACACAGCAAG
CATGTTGCAGTAATTCTTCAGCTGGTGACATGAACCATCGCAGGTTTAGGAAC
ATTTCAAGCACGAATGATTCATGGAAGGAAGTTTCCGAGGAGGTTGTAGTCT
ACCAGCATTGCCGAATTTCATTTCTATGTTACTCTTGAGCATCTATCACTGCTT
TTCTAATTCACATTTCTGCTGTCAGGGTCGTATGGCTTTCGATAAACTGTTCA
GTAGAGGAAAGCTTCCCCAAAGCTTTTCTCCTCCACAAGCAGAAGGATTGAA
GGTGGTTCCCAGAGGGGAGCAAGATGAAGCTACTACGGTGACGGTCGACCTC
AACAAGAGTGCTGCAGTTATGGACCATGAACTTGACACATTGGTTGGGCCAA
GAGCTTCCTTTCCCATTGAATTGTCACACCTGAATATGAAATCCCGCCGGACA
GGCTTCAAACCTTACAAGAGGTGCTCGGTGGAAGCAAAGGAGAATAGGGTG
CCGGCTGCTGACGAGGTTGGTACCAAAAGGATTCGCCTTGACAGCGAACCCT
CCACGTGATTTACTTCCCACGACATGATTACCAGCCTGCACAAGTAGTGTATT
TTCAAGAAATTGCGGTATTTACATCTAAGCTACTATAGGACTTGCCAGTCCTT
GCAATGCAGTG

Comparing the sequence of the *GIRNAi* construct with the barley genomic sequence of the *GI* gene

GI Forward primer

```
template        ----------------------------------------------------------TTCA 4
gi              ACCGCCAGCGATAGACATGTTTGTACAAAAAGCAGGCTCCGAATTCGCCCTTCACCTTCA 60
                                                                        ****

template        CTGAAGCGATGTAAGTGGGATGCTGAGATTTCTACCAGGGCATCATCCCTTTACCATTTG 64
gi              CTGAAGCGATGTAAGTGGGATGCTGAGATTTCTACCAGGGCATCATCCCTTTACCATTTG 120
                ************************************************************

template        ATCGATTTGCATGGTAAAACTGTGTCCTCCATCGTGAACAAAGCTGAGCCTCTAGAAGCT 124
gi              ATCGATTTGCATGGTAAAACTGTGTCCTCCATCGTGAACAAAGCTGAGCCTCTAGAAGCT 180
                ************************************************************

template        CACCTGACTTTTACATCAGTAAAGAGAGATGGTCAACAACACATTGAGGAAAACAGCACC 184
gi              CACCTGACTTTTACATCAGTAAAGAGAGATGGTCAACAACACATTGAGGAAAACAGCACC 240
                ************************************************************

template        AGCTCATCGGGTAATGGCAACTTGGAAAAGAAGAATGCTTCAGCCTCACACATGAAAAAT 244
gi              AGCTCATCGGGTAATGGCAACTTGGAAAAGAAGAATGCTTCAGCCTCACACATGAAAAAT 300
                ************************************************************

template        GGTTTTTCAAGACCACTCTTGAAATGCTCAGAAGAGGCTAGGCGAAATGGTAATGTTGCA 304
gi              GGTTTTTCAAGACCACTCTTGAAATGCTCAGAAGAGGCTAGGCGAAATGGTAATGTTGCA 360
                ************************************************************

template        AGTACATCCGGGAAAGTTCCTGCAACTTTACAGGCTGAAGCATCTGATTTGGCTAACTTC 364
gi              AGTACATCCGGGAAAGTTCCTGCAACTTTACAGGCTGAAGCATCTGATTTGGCTAACTTC 420
                ************************************************************

template        CTTACCATGGATAGAAATGGGGGTTATCGAGGCTCTCAG---------------------- 403
gi              CTTACCATGGATAGAAATGGGGGTTATCGAGGCTCTCAGAAGGGCGAATTCGACCCAGCT 480
                ************************************

template        ------------------------------------------------------------
gi              TTCTTGTACAAAGTGGTGATTAGAGCGGCCGCCACCGCGGTGGCTAGTGATTGATACCTG 540

template        ------------------------------------------------------------
gi              CACATCAACAAATTTTGGTCATATATTAGAAAAGTTATAAATTAAAATATACACACTTAT 600

template        ------------------------------------------------------------
gi              AAACTACAGAAAACCAATTGCTATATACTACATTCTTTTATTTTGAAAAAAATATTTGAA 660

template        ------------------------------------------------------------
gi              ATATTATATTACTACTAATTAATGAAATTATTATATATATATCAAAGGTACACCCACACT 720

template        ---------------------------------------
gi              TACGTACAATTGAATTGGGGGATCCTCGTGATTCAGCAA 759
```

GI Reverse primer

```
template        ------------------------------------------------------------
```

```
gi              CATTATCAAGACTAGAGTGGATCCCCCAATTCGAGCTCGCCCAATTCACTATCGAATTAT 60

template        ----------------------------------------TTCACTGAAGCGATGT 16
gi              CACAAGTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTCACCTTCACTGAAGCGATGT 120
                                                       ****************

template        AAGTGGGATGCTGAGATTTCTACCAGGGCATCATCCCTTTACCATTTGATCGATTTGCAT 76
gi              AAGTGGGATGCTGAGATTTCTACCAGGGCATCATCCCTTTACCATTTGATCGATTTGCAT 180
                ************************************************************

template        GGTAAAACTGTGTCCTCCATCGTGAACAAAGCTGAGCCTCTAGAAGCTCACCTGACTTTT 136
gi              GGTAAAACTGTGTCCTCCATCGTGAACAAAGCTGAGCCTCTAGAAGCTCACCTGACTTTT 240
                ************************************************************

template        ACATCAGTAAAGAGAGATGGTCAACAACACATTGAGGAAAACAGCACCAGCTCATCGGGT 196
gi              ACATCAGTAAAGAGAGATGGTCAACAACACATTGAGGAAAACAGCACCAGCTCATCGGGT 300
                ************************************************************

template        AATGGCAACTTGGAAAAGAAGAATGCTTCAGCCTCACACATGAAAAATGGTTTTTCAAGA 256
gi              AATGGCAACTTGGAAAAGAAGAATGCTTCAGCCTCACACATGAAAAATGGTTTTTCAAGA 360
                ************************************************************

template        CCACTCTTGAAATGCTCAGAAGAGGCTAGGCGAAATGGTAATGTTGCAAGTACATCCGGG 316
gi              CCACTCTTGAAATGCTCAGAAGAGGCTAGGCGAAATGGTAATGTTGCAAGTACATCCGGG 420
                ************************************************************

template        AAAGTTCCTGCAACTTTACAGGCTGAAGCATCTGATTTGGCTAACTTCCTTACCATGGAT 376
gi              AAAGTTCCTGCAACTTTACAGGCTGAAGCATCTGATTTGGCTAACTTCCTTACCATGGAT 480
                ************************************************************

template        AGAAATGGGGGTTATCGAGGCTCTCAG---------------------------------- 403
gi              AGAAATGGGGGTTATCGAGGCTCTCAGAAGGGCGAATTCGACCCAGCTTTCTTGTACAAA 540
                ****************************

template        ------------------------------------------------------------
gi              GTGGTGATTAGAGCGGCCGCCACCGCGGTGGCTAGAGGATCAATTCGATTCGGTCCTGAA 600

template        ------------------------------------------------------------
gi              TCACCAAAGATATGCAAAATGCATATAAATAAAACATTAAGCATTGGCGATTATTGCTCA 660

template        ------------------------------------------------------------
gi              GTTGTAGAATGAAGTAACTTTAAGAGTTGAAGACAATTGTTCCAGTGGCATACAACGATG 720

template        ------------------------------------------------------------
gi              AATCACTAGGATCCCCAATTCGATTGTACGTAAGTTCTGCTTCTACCTTTTGATATATAT 780

template        ------------------------------------------------------------
gi              ATAATAATTATCATTAATTAGTAGTAATATAATATTTCAAATATTTTTTTTCAAAATAAA 840

template        -------------------------------------------
gi              AGAATGCAGCATAAAGCCATTGCTTCCCTGTAGTCTACACTTT 883
```

Comparing the sequence of the *TOC1 RNAi* construct with the barley genomic sequence of the *TOC1* gene

TOC1 Forward primer

```
template        ----------------------------------------------------CACCAGGT 8
toc1            AGCCATTGGATCATCACAGTTTGTACAAAAAGCAGGCTCCGAATTCGCCCTTCACCAGGT 60
                                                                    ********

template        TAATTTCTCCGGTTCGACTGACAGAATCGACACTAATAGCAGTACAAATATTCAGGATGA 68
toc1            TAATTTCTCCGGTTGGACAGAAAAAATCGACACTAATAGCAGTACAAATATTCAGGATGA 120
                ************** *** ** * ***********************************

template        AAAAGCTTTTGAGATGCCCACGCAGTATCCTTTGGTTTGCTTTTCTTCCTCTAACTTGCA 128
toc1            AAAAGCTTTTGACATGCCCACGCAGTATCCTTTGGTTTGCTTTTCTTCCTCTAACTTGCA 180
                ************ *********************************************

template        ACTGGAGCAAAGGAATGAGGGTCAACAAGATGTTTCAGGAAACCCTCCTGTATATCATTA 188
toc1            TCTGGACCAAAGGAATGAGGGTCAACAAGATGTTTCAGGAAACCCTCCTGTATATCATTA 240
                 ***** ****************************************************

template        CCCATTTTATTATCCAGGGATGGTAGAGCATAGCATGGCACTTCATTCAGTTCAAAGTTT 248
toc1            CCCATTTTATTATCCAGGGATGGTAGAGT-TATTATGTCACTTCATTCAGTTCAAAGTTA 299
                ***************************  **  *** ********************

template        CCAAGGAAACATAAACACTGCTCAAGCTCATACACCACCAACAATGCTCCATCAATACAG 308
toc1            CCAAGGAAACATTCACACTGTTGCAGCTCATACACCACCTACCATGCTCCATCCATACAC 359
                ************  ****** *  *************** ** ********** *****

template        TGTTTATCACCAATCCCATGGTGCGTCTACAATGCCATCGTATCAGTACAATCCTGCTGG 368
toc1            GGATTCTCACCTATCCCATGGCGTGTCTACCCTGCCATCCTATCAATAACATCTTGCTGG 419
                 * ** ***** ********* * ******  ******* ***** **  *** ******

template        TATGAATGTGCATTCAAGTCACTTGTCAATGCAGAATGTGTGGTCATCGGTAT------- 421
toc1            CATGAATGTGCATTTAAGTCCCTTGGTTATGTTCAATGTGACGCCATCGTTAAAACGGCG 479
                 ************* ***** ****   ***   ******  * ***** **

template        ------------------------------------------------------------
toc1            AATTCTACCCAATTTTCTTGTACAAAGTGGTGATTAACGCCTCCGCCACCGTGGTATTAG 539


template        ------------------------------------------------------------
toc1            AGTTTCAATTTCATTCTGTCCTGCCTCACTAACATATTCGTAAATATTGAACATCTAACA 599


template        --
toc1            TT 601
```

TOC1 Reverse primer

```
template        ------------------------------------------------------------
toc1            CAATGGATTCTAGACTAGAGTGGATCCCCCAATTCGAGCTCGCCCAATTCACTATCGAAT 60


template        ----------------------------------------CACCAGGTTAATTTCTC 17
toc1            TATCACAAGTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTCACCAGGTTAATTTCTC 120
                                                        *****************

template        CGGTTCGACTGACAGAATCGACACTAATAGCAGTACAAATATTCAGGATGAAAAAGCTTT 77
toc1            CGGTTCGACTGACAGAATCGACACTAATAGCAGTACAAATATTCAGGATGAAAAAGCTTT 180
                ************************************************************
```

```
template        TGAGATGCCCACGCAGTATCCTTTGGTTTGCTTTTCTTCCTCTAACTTGCAACTGGAGCA 137
toc1            TGAGATGCCCACGCAGTATCCTTTGGTTTGCTTTTCTTCCTCTAACTTGCAACTGGAGCA 240
                ************************************************************

template        AAGGAATGAGGGTCAACAAGATGTTTCAGGAAACCCTCCTGTATATCATTACCCATTTTA 197
toc1            AAGGAATGAGGGTCAACAAGATGTTTCAGGAAACCCTCCTGTATATCATTACCCATTTTA 300
                ************************************************************

template        TTATCCAGGGATGGTAGAGCATAGCATGGCACTTCATTCAGTTCAAAGTTTCCAAGGAAA 257
toc1            TTATCCAGGGATGGTAGAGCATACCATGGCACTTCATTCAGTTCAAAGTTTCCAAGGAAA 360
                ********************** ***************************************

template        CATAAACACTGCTCAAGCTCATACACCACCAACAATGCTCCATCAATACAGTGTTTATCA 317
toc1            CATAAACACTGCTCAAGCTCATACACCACCAACAATGCTCCATCAATACAGTGTTTATCA 420
                ************************************************************

template        CCAATCCCATGGTGCGTCTACAATGCCATCGTATCAGTACAATCCTGCTGGTATGAATGT 377
toc1            CCAATCCCATGGTGCGTCTACAATGCCATCGGATCAGTACAATCCTGGTGGTATGAATGT 480
                ****************************** *************** ************

template        GCATTCAAGTCACTTGTCAATGCAGAATGTGTGGTCATCGGTAT---------------- 421
toc1            GCATTCAAGTCACTTGTCAATGCAGAATGTGAGGTCATCGGTATAAGGGCGAATTCGACC 540
                ****************************** ************

template        --------------------
toc1            CAGCTTTCTTGTACAAAGTGT 561
```

Comparing the sequence of the *CCA1 RNAi* entry vector construct with the barley genomic sequence of the *CCA1* gene

CCA1 Forward primer

```
template        ------------------------------------------------------------

cca1            GGGTTGTTCGACGGCAGTCTTAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAG 60


template        ------------------------------------------------------------

cca1            TGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACA 120


template        -------------------------CCAGTGCAACCAAGATGCCTACAGGTCATCTGTTG 35

cca1            AAAAAGCAGGCTCCGAATTCGCCCTTCAGTGCAACCAAGATGCCTACAGGTCATCTGTTG 180
                                         **********************************

template        ATATGTCGTCCACCTTCTCCAACATGCTCGTTTCCACATTGTTATCAAACCCCACAGTAC 95

cca1            ATATGTCGTCCACCTTCTCCAACATGCTCGTTTCCACATTGTTATCAAACCCCACAGTAC 240
                ************************************************************

template        ATGCAGCTGCAAGGCTTGCAGCATCATACTGGCCAGCAGCAGACAGCAACATCCCTGTCG 155

cca1            ATGCAGCTGCAAGGCTTGCAGCATCATACTGGCCAGCAGCAGGCAGCAACATCCCTGTCG 300
                ****************************************** ****************
```

```
template        ATCCAAATCAAGGAATTTTTGCTCAGAATGCTCAAGGAAGACATATTGTTTCTCCTCCAA 215
cca1            ATCCAAATCAAGGAATTTTTGCTCAGAATGCTCAAGGAAGACATATTGTTTCTCCTCCAA 360
                ************************************************************

template        GCATGGCTTCTGTGGTAGCAGCTACAGTTGCTGCGGCTTCGGCATGGTGGGCAACACAAG 275
cca1            GCATGGCTTCTGTGGTAGCAGCTACAGTTGCTGCGGCTTCGGCATGGTGGGCAACACAAG 420
                ************************************************************

template        GTCTTCTCCCTCTTTTTGCTCCCCCCATGGCTTTTCCATTTGTCCCAGTTCCTACCGCTT 335
cca1            GTCTTCTCCCTCTTTTTGCTCCCCCCATGGCTTTTCCATTTGTCCCAGTTCCTACCGCTT 480
                ************************************************************

template        CCTTTCCCACAGCGGATGTCCAGCGAGCTACAGAGAACTGCCCAGTGGACAACGCACCAA 395
cca1            CCTTTCCCACAGCGGATGTCCAGCGAGCTACAGAGAACTGCCCAGTGGACAACGCACCAA 540
                ************************************************************

template        AGGAATGCCAAGTAGCTCAGGGGCAAGGTCAACCTGAAGCTATGATAGTTGTAGCATCTT 455
cca1            AGGAATGCCAAGTAGCTCAGGGGCAAGGTCAACCTGAAGCTATGATAGTTGTAGCATCTT 600
                ************************************************************

template        CTGGGTCC-------------------------------------------------- 463
cca1            CTGGGTCCAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATA 660
                ********

template        ------------------------------------------------------------
cca1            ATTGCTCATCAATTAGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTT 720

template        ------------------------------------------------------------
cca1            GCCATCCAGCTGATATCCCCTATAGTGAGTCGTATTACATGGTCATAGCTGTTTCCTGGC 780

template        ------------------------------------------------------------
cca1            AGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAATATATATCAT 840

template        ----------------------------------------
cca1            CATGCCTCCTCCATACCAGCCCAGACAAAAAGCCTCGACT 880
```

CCA1Reverse primer

```
template        CCAGTGCAACCAAGATGCCTACAGGTCATCTGTTGATATGTCGTCCACCTTCTCCAACAT 60
cca1            ------------------------------------------------------------
```

```
template        GCTCGTTTCCACATTGTTATCAAACCCCACAGTACATGCAGCTGCAAGGCTTGCAGCATC 120
cca1            ------------------------------------------------------------

template        ATACTGGCCAGCAGCAGACAGCAACATCCCTGTCGATCCAAATCAAGGAATTTTTGCTCA 180
cca1            -------------GCAGGCAGCAACATACCTGTCGATCCAAATCAAGGAAATTTTGCTCA 47
                             **** ********* ********************** *********

template        GAATGCTCAAGGAAGACATATTGTTTCTCCTCCAAGCATGGCTTCTGTGGTAGCAGCTAC 240
cca1            GAATGCTCAAGGAAGACATATTGTTTCTCCTCCAAGCATGGCTTATGTGGTAGCAGCTAC 107
                ****************************************** ***************

template        AGTTGCTGCGGCTTCGGCATGGTGGGCAACACAAGGTCTTCTCCCTCTTTTTGCTCCCCC 300
cca1            AGTTGCTGCGGCTTTGGCATGGTGGGCAACACAAGGTCTTCTCCCTCTTTTTGCTCCCCC 167
                ************** ********************************************

template        CATGGCTTTTCCATTTGTCCCAGTTCCTACCGCTTCCTTTCCCACAGCGGATGTCCAGCG 360
cca1            CATGGCTTTTCCATTTGTCCCAGTTCCTACCGCTTCCTTTCCCACAGCGGATGTCCAGCG 227
                ************************************************************

template        AGCTACAGAGAACTGCCCAGTGGACAACGCACCAAAGGAATGCCAAGTAGCTCAGGGGCA 420
cca1            AGCTACAGAGAACTGCCCAGTGGACAACGCACCAAAGGAATGCCAAGTAGCTCAGGGGCA 287
                ************************************************************

template        AGGTCAACCTGAAGCTATGATAGTTGTAGCATCTTCTGGGTCC---------------- 463
cca1            AGGTCAACCTGAAGCTATGATAGTTGTAGCATCTTCTGGGTCCAAGGGCGAATTCGACCC 347
                ****************************************

template        ------------------------------------------------------------

cca1            AGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGCAGGGG 407

template        ------------------------------------------------------------

cca1            AACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGATATCCCCTATA 467

template        ---------------

cca1            ATAGTCGAATAATGA 482
```

Comparing the sequence of the *HvGI* construct with the barley genomic sequence of the *GI* gene

HvGI Forward primer

```
>template      1 ATGTCAGCGTCAAATGGGAAGTGGATTGATGGACTCCAGTTCTCATCACTGTTCTGGCCC   60
HvGI            ------------------------------------------------------------
>template     61 CCGCCACACGATGCGCAGCAGAAACAGGCACAAATTTTAGCCTATGTTGAGTACTTTGGT  120
HvGI            ------------------------------------------------------------
>template    121 CAGTTCACATCTGACAGCGAGCAATTCCCGGAGGATGTAGCTCAGCTAATTCAAACTTGC  180
HvGI            ------------------------------------------------------------
>template    181 TATCCATCAAAAGAAAAGCGGTTGGTAGATGAAGTATTAGCAACTTTTGTTCTCCATCAC  240
HvGI            ------------------------------------------------------------
>template    241 CCCGAGCATGGTCATGCAGTTGTACATCCAATTCTTTCACGCATCATAGACGGGACACTG  300
HvGI            ------------------------------------------------------------
>template    301 AGTTATGATAGTCATGGTTCCCCATTCAATTCCTTCATCTCCTTATTTACCCAAAGTTCT  360
HvGI            ------------------------------------------------------------
>template    361 GAGAAAGAGTACTCAGAGCAGTGGGCCTTGGCCTGTGGAGAGATTCTTAGAGTTCTAACT  420
HvGI            ------------------------------------------------------------
>template    421 CACTACAACAGGCCAATCTTTAAAGTTGCAGACTGTAACAACACCTCTGACCAGGCTACA  480
HvGI            ------------------------------------------------------------
>template    481 ACAAGTTGTTCTGCACAGGAGAAAGCTAATTACTCTCCAGGAAATGAACCTGAACGGAAG  540
HvGI            ------------------------------------------------------------
>template    541 CCATTGAGGCCATTATCTCCTTGGATCACAGATATTTTGCTAACTGCACCTTTAGGCATT  600
HvGI            ------------------------------------------------------------
>template    601 AGAAGCGACTATTTTAGATGGTGTGGTGGAGTTATGGGAAAATACGCAGCTGGTGGAGAA  660
HvGI            ------------------------------------------------------------
>template    661 TTGAAGCCTCCAACAACTGCTTACAGCCGAGGAGCTGGTAAGCACCCACAACTCATGCCA  720
HvGI            ------------------------------------------------------------
>template    721 TCCACCCCAAGATGGGCTGTTGCCAATGGAGCTGGAGTTATTTTAAGTGTCTGTGATGAG  780
HvGI            ------------------------------------------------------------
>template    781 GAAGTAGCTCGTTATGAGACAGCAAACTTAACCGCGGCAGCTGTTCCCGCCCTTCTGCTA  840
HvGI            ------------------------------------------------------------
>template    841 CCTCCACCGACAACACCCTTGGATGAGCATTTGGTGGCAGGGCTACCCCCTCTTGAGCCA  900
HvGI            ------------------------------------------------------------
```

```
>template    901 TACGCTCGCTTGTTTCATAGATACTATGCAATTGCCACACCAAGTGCTACACAAAGATTG  960
HvGI             ------------------------------------------------------------
>template    961 CTTTTTGGTCTTCTTGAAGCACCACCATCATGGGCTCCAGATGCACTTGATGCAGCAGTT 1020
HvGI             ------------------------------------------------------------
>template   1021 CAGCTTGTTGAACTCCTTCGGGCAGCTGAAGATTATGCTACTGGCATGCGGCTTCCAAAA 1080
HvGI             ------------------------------------------------------------
>template   1081 AATTGGTTGCATCTTCATTTCTTGCGTGCGATTGGAACTGCAATGTCTATGAGGGCTGGT 1140
HvGI             ------------------------------------------------------------
>template   1141 ATTGCTGCCGATACAGCTGCTGCGTTGCTTTTTCGCATACTATCCCAACCAACGTTGCTT 1200
HvGI             ------------------------------------------------------------
>template   1201 TTTCCTCCACTAAGGCATGCTGAAGGAGTTGAAGTGCAACATGAACCACTGGGTGGCTAT 1260
HvGI             ------------------------------------------------------------
>template   1261 GTATCATCATACAAAAGACAGCTGGAAGTTCCTGCATCTGAAACCACAATTGATGCCACT 1320
HvGI             ------------------------------------------------------------
>template   1321 GCACAAGGCATTGCTTCCTTGCTGTGTGCTCATGGTCCTGATGTTGAGTGGAGAATATGT 1380
HvGI             ------------------------------------------------------------
>template   1381 ACCATCTGGGAAGCTGCCTATGGTTTGTTACCTCTGAATTCATCAGCAGTTGATTTGCCC 1440
HvGI             ------------------------------------------------------------
>template   1441 GAAATCGTTGTAGCTGCTCCGCTTCAGCCACCTACTTTGTCATGGAGCCTATACTTGCCA 1500
HvGI             ------------------------------------------------------------
>template   1501 CTGTTGAAAGTATTCGAGTATCTACCTCGTGGAAGTCCATCTGAAGCATGCCTTATGAGA 1560
HvGI             ------------------------------------------------------------
>template   1561 ATATTTGTGGCAACAGTTGAAGCTATACTCAGAAGAACTTTCCCTTCGGAAACCTCTGAA 1620
HvGI             ------------------------------------------------------------
>template   1621 TCATCTAAAAGACCAAGAAGTCAATCCAAGAACCTTGCTGTTGCTGAACTCCGTACAATG 1680
HvGI             ------------------------------------------------------------
>template   1681 ATACATTCACTCTTTGTTGAATCATGTGCTTCAATGAACCTTGCTTCCCGGTTGTTGTTT 1740
HvGI             ------------------------------------------------------------
>template   1741 GTTGTATTAACTGTTTGCGTCAGTCATCAAGCTTTGCCAGGGGGCAGCAAAAGACCAACG 1800
HvGI             ------------------------------------------------------------
>template   1801 GGTAGTGAAAACCATTCTTCTGAGGAGGCCACTGAGGACCCAAGATTAACCAATGGAAGA 1860
HvGI             ------------------------------------------------------------
>template   1861 AATAAGGTCAAGAAGAAACAAGGGCCTGTTGGTACATTTGACTCGTATGTGCTGGCTGCT 1920
HvGI             ------------------------------------------------------------
```

```
>template   1921 GTTTGTGCCTTATCTTGTGAGCTTCAGCTGTTCCCTATCCTTTGCAAGAGTGCAACAAAC 1980
HvGI             ------------------------------------------------------------

>template   1981 TCAAAAGTAAAAGACTCTATAAAGATCCTGAAGCCTGGAAAAAACAATGGGATCAGTAAT 2040
HvGI             ------------------------------------------------------------

>template   2041 GAGCTACAGAATAGCATTAGCTCAGCAATTCTCCATACTCGTAGAATTCTTGGCATCCTG 2100
HvGI             ------------------------------------------------------------

>template   2101 GAAGCTCTTTTCTCCTTGAAGCCATCATCAGTTGGTACCTCCTGGAACTATAGTTCAAAT 2160
HvGI             ------------------------------------------------------------

>template   2161 GAGATAGTTGCAGCGGCTATGGTTGCCGCTCATGTTTCTGAGTTATTTCGCCGGTCGAGG 2220
HvGI             ------------------------------------------------------------

>template   2221 CCATGCCTAAATGCACTATCTTCACTGAAGCGATGTAAGTGGGATGCTGAGATTTCTACC 2280
HvGI             ------------------------------------------------------------

>template   2281 AGGGCATCATCCCTTTACCATTTGATCGATTTGCATGGTAAAACTGTGTCCTCCATCGTG 2340
HvGI             ------------------------------------------------------------

>template   2341 AACAAAGCTGAGCCTCTAGAAGCTCACCTGACTTTTACATCAGTAAAGAGAGATGGTCAA 2400
HvGI             ------------------------------------------------------------

>template   2401 CAACACATTGAGGAAAACAGCACCAGCTCATCGGGTAATGGCAACTTGGAAAAGAAGAAT 2460
HvGI             ------------------------------------------------------------

>template   2461 GCTTCAGCCTCACACATGAAAAATGGTTTTTCAAGACCACTCTTGAAATGCTCAGAAGAG 2520
HvGI             ------------------------------------------------------------

>template   2521 GCTAGGCGAAATGGTAATGTTGCAAGTACATCCGGGAAAGTTCCTGCAACTTTACAGGCT 2580
HvGI             ------------------------------------------------------------

>template   2581 GAAGCATCTGATTTGGCTAACTTCCTTACCATGGATAGAAATGGGGGTTATCGAGGCTCT 2640
HvGI             ------------------------------------------------------------

>template   2641 CAGACTCTCCTAAGTTCTGTTATCTCAGAAAAACAGGAATTATGCTTCTCTGTTGTCTCA 2700
                                          T AGAA A CA GAA TATGC TCTC GTTGT  CA
HvGI           1 -------------------------TGAGAAGAGCA-GAAGTATGCCTCTC-GTTGTTCCA   34

>template   2701 TTGCTCTGGCATAAGCTT-ATTGCATCTCCTGAAACGCAGATGTCT-GCA-GAAAGTACA 2757
                 T GCTC  GC TA  CTT AT GCAT TCCTG A   CAGATGT T GCA GAAAGTACA
HvGI          35 TAGCTC--GC-TAGCCTTAATGGCAT-TCCTGGATGCCAGATGTTTGGCAGGAAAGTACA   90

>template   2758 TCAGCTC-ATCAAGGTTGGAGAAAGGTTGTAG--ATGCCCTTTG-TGATGTTGTTTCAGC 2813
                 TCAGCTC ATC A G TG AGAAA G TGTAG   TGCC TTTG TGATGTTGTT CAGC
HvGI          91 TCAGCTCCATCCATGATG-AGAAACGATGTAGGATTGCCTTTTGTTGATGTTGTTCCAGC  149

>template   2814 CT-CACCAGC-CAAGG-CTTCAA-CTGCTATTGTTCTGCA-GGCTGAGAAGGACCTACAG 2868
                 CT CACCAGC CAAGG CTTCAA CTGCTATTGTTCTGCA GGCTGAGA GGACCTACAG
HvGI         150 CTCCACCAGCCCAAGGCCTTCAACCTGCTATTGTTCTGCAGGGCTGAGAGGGACCTACAG  209

>template   2869 C-CCTGGATTGCTCGAGATGACGAGG-AAGGTCAAAAGATGTGGAGAGTCAA-CCAGCGA 2925
                 C CCTGGATTGCTCGAGATGACGAGG AAGGTCAAAAGATGTGGAGAGTCAA CCAGCGA
HvGI         210 CCCCTGGATTGCTCGAGATGACGAGGAAAGGTCAAAAGATGTGGAGAGTCAACCCAGCGA  269
```

```
>template   2926 ATAGTTAAAC-TGATAGCTGAGCTTATGAGGAACCATGATAGCCCAGAA-GCATTGATAA 2983
                 ATAGTTAAAC TGATAGCTGAGCTTATGAGGAACCATGATAGCCCAGAA GCATTGATAA
HvGI         270 ATAGTTAAACCTGATAGCTGAGCTTATGAGGAACCATGATAGCCCAGAACGCATTGATAA  329

>template   2984 TTCTTGCTAGTGCTTCAGACCTTCTGCTTCGTGCTACAGATGGGATGCTTGTTGATGGTG 3043
                 TTCTTGCTAGTGCTTCAGACCTTCTGCTTCGTGCTACAGATGGGATGCTTGTTGATGGTG
HvGI         330 TTCTTGCTAGTGCTTCAGACCTTCTGCTTCGTGCTACAGATGGGATGCTTGTTGATGGTG  389

>template   3044 AAGCTTGTACCTTGCCTCAGTTAGAGCTCCTGGAAGTAACTGCTAGAGCTATTCATCTCA 3103
                 AAGCTTGTACCTTGCCTCAGTTAGAGCTCCTGGAAGTAACTGCTAGAGCTATTCATCTCA
HvGI         390 AAGCTTGTACCTTGCCTCAGTTAGAGCTCCTGGAAGTAACTGCTAGAGCTATTCATCTCA  449

>template   3104 TCGTTGAATGGGGAGACCCAGGTGTAGCAGTTGCTGATGGCCTCTCAAATCTGTTAAAGT 3163
                 TCGTTGAATGGGGAGACCCAGGTGTAGCAGTTGCTGATGGCCTCTCAAATCTGTTAAAGT
HvGI         450 TCGTTGAATGGGGAGACCCAGGTGTAGCAGTTGCTGATGGCCTCTCAAATCTGTTAAAGT  509

>template   3164 GCCGTCTATCGCCTACCATCCGCTGCCTCTCCCACGCTAGCGCACACGTACGAGCGCTCA 3223
                 GCCGTCTATCGCCTACCATCCGCTGCCTCTCCCACGCTAGCGCACACGTACGAGCGCTCA
HvGI         510 GCCGTCTATCGCCTACCATCCGCTGCCTCTCCCACGCTAGCGCACACGTACGAGCGCTCA  569

>template   3224 GCATGTCCGTCCTCCGTGACATCTTGAACAGTGGACCACTAGGTTCCAGTAAGACCATTC 3283
                 GCATGTCCGTCCTCCGTGACATCTTGAACAGTGGACCACTAGGTTCCAGTAAGACCATTC
HvGI         570 GCATGTCCGTCCTCCGTGACATCTTGAACAGTGGACCACTAGGTTCCAGTAAGACCATTC  629

>template   3284 AAGGCGAGCAACGGAACGGCATCCAAAGCCCAAACTACCAATGCGCGGCAGCAAACACGG 3343
                 AAGGCGAGCAACGGAACGGCATCCAAAGCCCAAACTACCAATGCGCGGCAGCAAACACGG
HvGI         630 AAGGCGAGCAACGGAACGGCATCCAAAGCCCAAACTACCAATGCGCGGCAGCAAACACGG  689

>template   3344 TGAACTGGCAAGCGGATGTCGAGAGATGTATAGACTGGGAAGCCCGCAGCCGCCGTGCCA 3403
                 TGAACTGGCAAGCGGATGTCGAGAGATGTATAGACTGGGAAGCCCGCAGCCGCCGTGCCA
HvGI         690 TGAACTGGCAAGCGGATGTCGAGAGATGTATAGACTGGGAAGCCCGCAGCCGCCGTGCCA  749

>template   3404 CCGGGATGACCCTCGCCTTCCTCACCGCTGCTGCTAACGAGCTCGGCTGCCCCCTTCCTT 3463
                 CCGGGATGACCCTCGCCTTCCTCACCGCTGCTGCTAACGAGCTCGGCTGCCCCCTTCCTT
HvGI         750 CCGGGATGACCCTCGCCTTCCTCACCGCTGCTGCTAACGAGCTCGGCTGCCCCCTTCCTT  809

>template   3464 GCTGACAAGGCCATATT---------------------------------------------- 3480
                 GCTGACAAGGCCATATT
HvGI         810 GCTGACAAGGCCATATTTGAAGCTGACAATCAGCAACACTTGACAGTTGGTGCGAGCAGT  869

>template        ------------------------------------------------------------

HvGI         870 TGCTGCATGGTCAGCGAGCAGGATGGCTAATTAATCCCTTGCTCAAGGATGGCTTTCCAT  929

>template        ------------------------------------------------------------

HvGI         930 TCCCAGTCTGCCCCCCATGATGTGATGTATATTAGCTGATGTTCCCAATCATACGGAGCT  989

>template        ------------------------------------------------------------

HvGI         990 TTGCTCCCGCGGTGTGATTTTAACTTGCAATCTGACGTTAGATGTTCAAGCATATTGAAC 1049

>template        ------------------------------------------------------------

HvGI        1050 TGCTTGTGCTGTAAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTGGTTCGATA 1109

>template        ------------------------------------------------------------

HvGI        1110 ATTCTTAATTAACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCGAATTTCCCCGAT 1169

>template        ----------------------------------

HvGI        1170 CGTCAAACATTTGGCAAATAAAAATTTAAAGGT 1202
```

HvGI Reverse primer

```
>template    1 ATGTCAGCGTCAAATGGGAAGTGGATTGATGGACTCCAGTTCTCATCACTGTTCTGGCCC   60
hvgi           ------------------------------------------------------------
>template   61 CCGCCACACGATGCGCAGCAGAAACAGGCACAAATTTTAGCCTATGTTGAGTACTTTGGT  120
hvgi           ------------------------------------------------------------
>template  121 CAGTTCACATCTGACAGCGAGCAATTCCCGGAGGATGTAGCTCAGCTAATTCAAACTTGC  180
hvgi           ------------------------------------------------------------
>template  181 TATCCATCAAAAGAAAAGCGGTTGGTAGATGAAGTATTAGCAACTTTTGTTCTCCATCAC  240
hvgi           ------------------------------------------------------------
>template  241 CCCGAGCATGGTCATGCAGTTGTACATCCAATTCTTTCACGCATCATAGACGGGACACTG  300
hvgi           ------------------------------------------------------------
>template  301 AGTTATGATAGTCATGGTTCCCCATTCAATTCCTTCATCTCCTTATTTACCCAAAGTTCT  360
hvgi           ------------------------------------------------------------
>template  361 GAGAAAGAGTACTCAGAGCAGTGGGCCTTGGCCTGTGGAGAGATTCTTAGAGTTCTAACT  420
hvgi           ------------------------------------------------------------
>template  421 CACTACAACAGGCCAATCTTTAAAGTTGCAGACTGTAACAACACCTCTGACCAGGCTACA  480
hvgi           ------------------------------------------------------------
>template  481 ACAAGTTGTTCTGCACAGGAGAAAGCTAATTACTCTCCAGGAAATGAACCTGAACGGAAG  540
hvgi           ------------------------------------------------------------
>template  541 CCATTGAGGCCATTATCTCCTTGGATCACAGATATTTTGCTAACTGCACCTTTAGGCATT  600
hvgi           ------------------------------------------------------------
>template  601 AGAAGCGACTATTTTAGATGGTGTGGTGGAGTTATGGGAAAATACGCAGCTGGTGGAGAA  660
hvgi           ------------------------------------------------------------
>template  661 TTGAAGCCTCCAACAACTGCTTACAGCCGAGGAGCTGGTAAGCACCCACAACTCATGCCA  720
hvgi           ------------------------------------------------------------
>template  721 TCCACCCCAAGATGGGCTGTTGCCAATGGAGCTGGAGTTATTTTAAGTGTCTGTGATGAG  780
hvgi           ------------------------------------------------------------
>template  781 GAAGTAGCTCGTTATGAGACAGCAAACTTAACCGCGGCAGCTGTTCCCGCCCTTCTGCTA  840
hvgi           ------------------------------------------------------------
>template  841 CCTCCACCGACAACACCCTTGGATGAGCATTTGGTGGCAGGGCTACCCCCTCTTGAGCCA  900
hvgi           ------------------------------------------------------------
>template  901 TACGCTCGCTTGTTTCATAGATACTATGCAATTGCCACACCAAGTGCTACACAAAGATTG  960
hvgi           ------------------------------------------------------------
```

```
>template  961 CTTTTTGGTCTTCTTGAAGCACCACCATCATGGGCTCCAGATGCACTTGATGCAGCAGTT 1020
hvgi            ------------------------------------------------------------
>template 1021 CAGCTTGTTGAACTCCTTCGGGCAGCTGAAGATTATGCTACTGGCATGCGGCTTCCAAAA 1080
hvgi            ------------------------------------------------------------
>template 1081 AATTGGTTGCATCTTCATTTCTTGCGTGCGATTGGAACTGCAATGTCTATGAGGGCTGGT 1140
hvgi            ------------------------------------------------------------
>template 1141 ATTGCTGCCGATACAGCTGCTGCGTTGCTTTTTCGCATACTATCCCAACCAACGTTGCTT 1200
hvgi            ------------------------------------------------------------
>template 1201 TTTCCTCCACTAAGGCATGCTGAAGGAGTTGAAGTGCAACATGAACCACTGGGTGGCTAT 1260
hvgi            ------------------------------------------------------------
>template 1261 GTATCATCATACAAAAGACAGCTGGAAGTTCCTGCATCTGAAACCACAATTGATGCCACT 1320
hvgi            ------------------------------------------------------------
>template 1321 GCACAAGGCATTGCTTCCTTGCTGTGTGCTCATGGTCCTGATGTTGAGTGGAGAATATGT 1380
hvgi            ------------------------------------------------------------
>template 1381 ACCATCTGGGAAGCTGCCTATGGTTTGTTACCTCTGAATTCATCAGCAGTTGATTTGCCC 1440
hvgi            ------------------------------------------------------------
>template 1441 GAAATCGTTGTAGCTGCTCCGCTTCAGCCACCTACTTTGTCATGGAGCCTATACTTGCCA 1500
hvgi            ------------------------------------------------------------
>template 1501 CTGTTGAAAGTATTCGAGTATCTACCTCGTGGAAGTCCATCTGAAGCATGCCTTATGAGA 1560
hvgi            ------------------------------------------------------------
>template 1561 ATATTTGTGGCAACAGTTGAAGCTATACTCAGAAGAACTTTCCCTTCGGAAACCTCTGAA 1620
hvgi            ------------------------------------------------------------
>template 1621 TCATCTAAAAGACCAAGAAGTCAATCCAAGAACCTTGCTGTTGCTGAACTCCGTACAATG 1680
hvgi            ------------------------------------------------------------
>template 1681 ATACATTCACTCTTTGTTGAATCATGTGCTTCAATGAACCTTGCTTCCCGGTTGTTGTTT 1740
hvgi            ------------------------------------------------------------
>template 1741 GTTGTATTAACTGTTTGCGTCAGTCATCAAGCTTTGCCAGGGGGCAGCAAAAGACCAACG 1800
hvgi            ------------------------------------------------------------
>template 1801 GGTAGTGAAAACCATTCTTCTGAGGAGGCCACTGAGGACCCAAGATTAACCAATGGAAGA 1860
hvgi            ------------------------------------------------------------
>template 1861 AATAAGGTCAAGAAGAAACAAGGGCCTGTTGGTACATTTGACTCGTATGTGCTGGCTGCT 1920
hvgi            ------------------------------------------------------------
>template 1921 GTTTGTGCCTTATCTTGTGAGCTTCAGCTGTTCCCTATCCTTTGCAAGAGTGCAACAAAC 1980
hvgi            ------------------------------------------------------------
```

>template 1981 TCAAAAGTAAAAGACTCTATAAAGATCCTGAAGCCTGGAAAAAACAATGGGATCAGTAAT 2040

hvgi ----------

>template 2041 GAGCTACAGAATAGCATTAGCTCAGCAATTCTCCATACTCGTAGAATTCTTGGCATCCTG 2100

hvgi ----------

>template 2101 GAAGCTCTTTTCTCCTTGAAGCCATCATCAGTTGGTACCTCCTGGAACTATAGTTCAAAT 2160

hvgi ----------

>template 2161 GAGATAGTTGCAGCGGCTATGGTTGCCGCTCATGTTTCTGAGTTATTTCGCCGGTCGAGG 2220

hvgi ----------

>template 2221 CCATGCCTAAATGCACTATCTTCACTGAAGCGATGTAAGTGGGATGCTGAGATTTCTACC 2280

hvgi ----------

>template 2281 AGGGCATCATCCCTTTACCATTTGATCGATTTGCATGGTAAAACTGTGTCCTCCATCGTG 2340

hvgi ----------

>template 2341 AACAAAGCTGAGCCTCTAGAAGCTCACCTGACTTTTACATCAGTAAAGAGAGATGGTCAA 2400

hvgi ----------

>template 2401 CAACACATTGAGGAAAACAGCACCAGCTCATCGGGTAATGGCAACTTGGAAAAGAAGAAT 2460

hvgi ----------

>template 2461 GCTTCAGCCTCACACATGAAAAATGGTTTTTCAAGACCACTCTTGAAATGCTCAGAAGAG 2520

hvgi ----------

>template 2521 GCTAGGCGAAATGGTAATGTTGCAAGTACATCCGGGAAAGTTCCTGCAACTTTACAGGCT 2580

hvgi ----------

>template 2581 GAAGCATCTGATTTGGCTAACTTCCTTACCATGGATAGAAATGGGGGTTATCGAGGCTCT 2640

hvgi ----------

>template 2641 CAGACTCTCCTAAGTTCTGTTATCTCAGAAAAACAGGAATTATGCTTCTCTGTTGTCTCA 2700

hvgi ----------

>template 2701 TTGCTCTGGCATAAGCTTATTGCATCTCCTGAAACGCAGATGTCTGCAGAAAGTACATCA 2760

hvgi ----------

>template 2761 GCTCATCAAGGTTGGAGAAAGGTTGTAGATGCCCTTTGTGATGTTGTTTCAGCCTCACCA 2820

hvgi ----------

>template 2821 GCCAAGGCTTCAACTGCTATTGTTCTGCAGGCTGAGAAGGACCTACAGCCCTGGATTGCT 2880

hvgi ----------

>template 2881 CGAGATGACGAGGAAGGTCAAAAGATGTGGAGAGTCAACCAGCGAATAGTTAAACTGATA 2940

hvgi ----------

>template 2941 GCTGAGCTTATGAGGAACCATGATAGCCCAGAAGCATTGATAATTCTTGCTAGTGCTTCA 3000

hvgi ----------

```
>template 3001 GACCTTCTGCTTCGTGCTACAGATGGGATGCTTGTTGATGGTGAAGCTTGTACCTTGCCT 3060

hvgi            ------------------------------------------------------------

>template 3061 CAGTTAGAGCTCCTGGAAGTAACTGCTAGAGCTATTCATCTCATCGTTGAATGGGGAGAC 3120

hvgi            ------------------------------------------------------------

>template 3121 CCAGGTGTAGCAGTTGCTGATGGCCTCTCAAATCTGTTAAAGTGCCGTCTATCGCCTACC 3180

hvgi            ------------------------------------------------------------

>template 3181 ATCCGCTGCCTCTCCCACGCTAGCGCACACGTACGA-GCGCTCAGCATGTCCGTCCTCCG 3239
                                 G GCA A  TACGA GCGCTCAGCATGTCCGTCCTCCG
hvgi         1 ----------------------GGGCATATCTACGAGGCGCTCAGCATGTCCGTCCTCCG   38

>template 3240 TGACATCTTGAACAGTGGACCACTAGGTTCCAGTAAGACCATTCAAGGCGAGCAACGGAA 3299
               TGACATCTTGAACAGTGGACCACTAGGTTCCAGTAAGACCATTCAAGGCGAGCAACGGAA
hvgi        39 TGACATCTTGAACAGTGGACCACTAGGTTCCAGTAAGACCATTCAAGGCGAGCAACGGAA   98

>template 3300 CGGCATCCAAAGCCCAAACTACCAATGCGCGGCAGCAAACACGGTGAACTGGCAAGCGGA 3359
               CGGCATCCAAAGCCCAAACTACCAATGCGCGGCAGCAAACACGGTGAACTGGCAAGCGGA
hvgi        99 CGGCATCCAAAGCCCAAACTACCAATGCGCGGCAGCAAACACGGTGAACTGGCAAGCGGA  158

>template 3360 TGTCGAGAGATGTATAGACTGGGAAGCCCGCAGCCGCCGTGCCACCGGGATGACCCTCGC 3419
               TGTCGAGAGATGTATAGACTGGGAAGCCCGCAGCCGCCGTGCCACCGGGATGACCCTCGC
hvgi       159 TGTCGAGAGATGTATAGACTGGGAAGCCCGCAGCCGCCGTGCCACCGGGATGACCCTCGC  218

>template 3420 CTTCCTCACCGCTGCTGCTAACGAGCTCGGCTGCCCCCTTCCTTGCTGACAAGGCCATAT 3479
               CTTCCTCACCGCTGCTGCTAACGAGCTCGGCTGCCCCCTTCCTTGCTGACAAGGCCATAT
hvgi       219 CTTCCTCACCGCTGCTGCTAACGAGCTCGGCTGCCCCCTTCCTTGCTGACAAGGCCATAT  278

>template 3480 T---------------------------------------------------------- 3480
               T
hvgi       279 TTGAAGCTGACAATCAGCAACACTTGACAGTTGGTGCGAGCAGTTGCTGCATGGTCAGCG  338

>template     ------------------------------------------------------------

hvgi       339 AGCAGGATGGCTAATTAATCCCTTGCTCAAGGATGGCTTTCCATTCCCAGTCTGCCCCCC  398

>template      ------------------------------------------------------------

hvgi       399 ATGATGTGATGTATATTAGCTGATGTTCCCAATCATACGGAGCTTTGCTCCCGCGGTGTG  458

>template      ------------------------------------------------------------

hvgi       459 ATTTTAACTTGCAATCTGACGTTAGATGTTCAAGCATATTGAACTGCTTGTGCTGTAAAG  518

>template      ------------------------------------------------------------

hvgi       519 GGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTGGTTCGATAATTCTTAATTAACTAG  578

>template       ------------------------------------------------------------

hvgi       579 TTCTAGAGCGGCCGCCACCGCGGTGGAGCTCGAATTTCCCCGATCGTTCAAACATTTGGC  638

>template      ------------------------------------------------------------

hvgi       639 AATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTC  698

>template       ------------------------------------------------------------

hvgi       699 TGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGAT  758

>template      ------------------------------------------------------------

hvgi       759 GGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATAT  818
```

```
>template      ------------------------------------------------------------
hvgi       819 AGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAAT  878
>template    ------------------------------------------------------------
hvgi       879 TCGTAATCATGTCATAGCTGTTTCCTGTGTGAAATTGTATCCGCTCACAATTCCACACAA  938
>template      ------------------------------------------------------------
hvgi       939 CAATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGGTGCTTAATGAGTGAGCTACTCC  998
>template      ------------------------------------------------------------
hvgi       999 ACATAATTGCGTTGCGCCTCACTTGCGGCTTTCAGTCCGGAACTGTCTGCAGCTGCATAT 1058
>template    ------------------------------------------------------------
hvgi      1059 GATCGCACGCGCGGGAAGGCGATGCCTATGGCATAGCACTTGCACATGTGGAACTGAACC 1118
>template     -------------------------------------------------
hvgi      1119 TCGTCTATCCCGAATCTAGATCGTACGGAGACAGGCATTGACCTCGACAG 1168
```

APPENDIX 2: ANOVA Tables

Appendix Table 2.1. Dry matter production of three barley genotypes that were under different T cycles and temperature.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	0.12730IJKLMN	0.1236IJKLMNO	0.09635LMNOPQ	0.2113CD
	PDX	0.10140JKLMNOPQ	0.1006KLMNOPQ	0.08725NOPQ	0.19475DEF
	GP	0.06885Q	0.08365OPQ	0.07505PQ	0.1697EFGH
22°C	PDWT	0.17155DEFG	0.13825GHIJKL	0.1224NOPQ	0.31075A
	PDX	0.16410FGHI	0.11125JKLMNOP	0.1123JKLMNOP	0.2699AB
	GP	0.14270GHIJ	0.09135MNOPQ	0.09425MNOPQ	0.2019CDEF
27°C	PDWT	0.13785GHIJK	0.12898HIJKLM	0.1139JKLMNOP	0.2391BC
	PDX	0.11875JKLMNO	0.11858JKLMNO	0.08745 OPQ	0.27455AB
	GP	0.10195JKLMNOPQ	0.09185MNOPQ	0.0751PQ	0.2072CDE
LSD (interaction) α0.05 =0.026399					

Appendix Table 2.2. Two-way ANOVA for dry matter production.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	34.21	11.402	1002.1**	2.61
temperature	2	2.13	1.063	93.42**	3.00
Type	2	4.15	2.077	182.58**	3.00
Time	1	0.53	0.535	46.98**	3.85
Light dark cycle* temperature	6	0.34	0.057	4.995**	2.11
Light dark cycle* Type	6	0.25	0.041	3.59**	2.11
Temperature* type	4	0.15	0.038	3.33*	2.38
Light dark cycle* Time	3	1.65	0.549	48.23**	2.61
Temperature* Time	2	2.49	1.245	109.45**	3.00
Type*time	2	0.07	0.034	3.00	3.00
Light-dark cycle*temperature*type	12	0.53	0.044	3.91**	1.76
Light dark cycle* temperature* Time	6	2.01	0.334	29.4**	2.11
Light dark cycle* Type* Time	6	0.08	0.013	1.11	2.11
Temperature* type* time	4	0.18	0.046	4.02**	2.38
Light dark cycle* temperature* type*time	12	0.39	0.032	2.85**	1.76
Block	1	0.06	0.058	5.073*	3.85
Error	1367	15.55	0.011		

Appendix Table 2.3. Fresh matter production of three barley genotypes grown under different T cycles and temperatures.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	1.1569EFGHI	0.9257HIJKL	0.8546IJKL	1.4838CDE
	PDX	0.9882HIJKL	0.8213IJKL	0.7812KL	1.3911CDEF
	GP	0.7708KL	0.7901JKL	0.6899L	1.2442DEFGH
22°C	PDWT	1.1405EFGHIJ	1.1156FGHIJK	0.9589HIJKL	2.0418A
	PDX	0.8872IJKL	0.9382HIJKL	0.8936HIJKL	1.8391AB
	GP	0.7094L	0.8845IJKL	0.7532L	1.6643BC
27°C	PDWT	1.1601EFGHI	1.0092GHIJKL	0.8887IJKL	1.1584EFGHI
	PDX	0.9552HIJKL	0.9685HIJKL	0.7643KL	1.5508BCD
	GP	0.8721IJKL	0.8630IJKL	0.6811L	1.348CDEFG
LSD (interaction)α0.05 =0.224813					

Appendix Table 2.4. Two-way ANOVA for fresh matter production.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	100.12	33.39	360.21**	2.61
temperature	2	2.61	1.30	14.07**	3.00
Type	2	9.71	4,85	52.37**	3.00
Time	1	4.44	4.44	47.93**	3.85
Light dark cycle* temperature	6	8.89	1.48	15.99**	2.11
Light dark cycle* Type	6	1.46	0.24	2.63*	2.11
Temperature* type	4	3.90	0.98	10.53**	2.38
Light dark cycle* Time	3	4.89	1.63	17.57**	2.61
Temperature* Time	2	15.77	7.89	85.08**	3.00
Type*time	2	0.03	0.02	0.181	3.00
Light dark cycle* temperature* type	12	9.86	0.82	8.87**	1.76
Light dark cycle* temperature* Time	6	20.67	3.45	37.17**	2.11
Light dark cycle* Type* Time	6	1.29	0.22	2.32*	2.11
Temperature* type* time	4	0.43	0.11	1.17	2.38
Light dark cycle* temperature* type* time	12	2.63	0.22	2.36**	1.76
Block	1	3.52	3.52	37.93**	3.85
Error	1367	126.72	0.09		

Appendix Table 2.5. Average plant length of three barley genotypes grown under different T cycles and temperature after 15 days.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	13.15EFGH	13.046EFGH	11.857FGHIJK	13.058EFGH
	PDX	11.366GHIJKLM	11.668GHIJKL	11.04HIJKLM	12.198FGHIJ
	GP	9.001MN	9.669JKLM	9.275LMN	9.978IJKLM
22°C	PDWT	12.344FGHI	14,382BCDEF	12.522FGHI	16.13ABCD
	PDX	12.314FGHI	12.165GHIJK	11.377GHIJKLM	13.626DEFG
	GP	9.372KLMN	10.439IJKLM	7.039N	11.657GHIJKL
27°C	PDWT	16.478AB	16.312ABC	16.485AB	16.92AB
	PDX	15.241ABCDE	17.056 A	15.478ABCDE	17.296A
	GP	11.962FGHIJ	12.007FGHIJ	12.179FGHIJ	13.87CDEFG
LSD (interaction) α0.05 = 1.632516					

Appendix Table 2.6. Two-way ANOVA for average plant height after 15 days.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	242	81	61.27**	2.61
temperature	2	1655	827	627.98**	3.00
Type	2	895	448	339.65**	3.00
Time	1	8789	8789	6670.4**	3.85
Light dark cycle* temperature	6	63	10	7.93**	2.11
Light dark cycle* Type	6	12	2	1.46	2.11
Temperature* type	4	39	10	7.42**	2.38
Light dark cycle* Time	3	20	7	5.07**	2.61
Temperature* Time	2	6	3	2.29	3.00
Type*time	2	123	61	46.67**	3.00
Light dark cycle* temperature* type	12	72	6	4.54**	1.76
Light dark cycle* temperature* Time	6	11	2	1.36	2.11
Light dark cycle* Type* Time	6	6	1	0.804	2.11
Temperature* type* time	4	10	2	1.86	2.38
Light dark cycle* temperature* type* time	12	12	1	0.78	1.76
Block	1	12	12	8.89**	3.85
Error	359	473	1		

Appendix Table 2.7. Total chlorophyll content of three barley genotypes grown under different T cycles and temperature after 15 days.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	1.8686CDEFGH	1.5865EFGH	1.3874FGH	1.6856DEFGH
	PDX	1.6321DEFGH	1.8408ABCDEFGH	1.884GH	2.8828AB
	GP	1.665DEFGH	1.6437DEFGH	1.647H	1.7548DEFGH
22°C	PDWT	2.2883ABCDEFG	2.2036ABCDEFGH	2.2391ABCDEFGH	2.4099ABCDE
	PDX	1.974CDEFGH	2.5091ABCD	2.4186ABCDE	2.9197A
	GP	1.9054CDEFGH	1.9104CDEFGH	2.425ABCDE	2.3804ABCDEF
27°C	PDWT	2.0845ABCDEFGH	2.2694ABCDEFG	1.5114FGH	2.4322ABCDE
	PDX	2.0475ABCDEFGH	2.2866ABCDEFG	1.6518DEFGH	2.7007ABC
	GP	2.0281BCDEFGH	2.2765ABCDEFG	1.5474EFGH	1.7315DEFGH
LSD (interaction) α0.05 = 0.554372					

Appendix Table 2.8. Two-way ANOVA for total chlorophyll content after 15 days.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	9.64	3.215	27.65**	2.61
temperature	2	10.93	5.463	46.99**	3.00
Type	2	5.16	2.578	22.17**	3.00
Time	1	0.08	0.08	0.72	3.85
Light dark cycle* temperature	6	7.04	1.174	10.09**	2.11
Light dark cycle* Type	6	7.34	1.224	10.53**	2.11
Temperature* type	4	0.96	0.24	2.06	2.38
Light dark cycle* Time	3	0.55	0.183	1.57	2.61
Temperature* Time	2	0.74	0.368	3.17*	3.00
Type*time	2	0.07	0.033	0.28	3.00
Light dark cycle* temperature* type	12	4.04	0.336	2.89**	1.76
Light dark cycle* temperature* Time	6	0.38	0.064	0.55	2.11
Light dark cycle* Type* Time	6	0.36	0.060	0.52	2.11
Temperature* type* time	4	0.12	0.030	0.26	2.38
Light dark cycle* temperature* type* time	12	0.28	0.023	0.199	1.76
Block	1	0.14	0.14	1.24	3.85
Error	287	33.37	0.116		

Appendix Table 2.9. Relative growth rates (RGR) of three barley genotypes grown under different T cycles and temperatures.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	0.14631ABCDEF	0.14023BCDEF	0.14583ABCDEF	0.23122AB
	PDX	0.11951CDEF	0.12277CDEF	0.10449CDEF	0.18595ABCDE
	GP	0.11067CDEF	0.1193CDEF	0.14686ABCDEF	0.14523ABCDEF
22°C	PDWT	0.11453CDEF	0.18201ABCDE	0.15581ABCDEF	0.24523A
	PDX	0.10485CDEF	0.14236ABCDEF	0.09516DEF	0.19349ABCD
	GP	0.08937EF	0.11485CDEF	0.05593F	0.19195ABCDE
27°C	PDWT	0.16657ABCDE	0.16862ABCDE	0.15512ABCDEF	0.17417ABCDE
	PDX	0.11695CDEF	0.15331ABCDEF	0.13023BCDEF	0.20758ABC
	GP	0.14943ABCDEF	0.11945CDEF	0.13001BCDEF	0.17664ABCDE
LSD (interaction) α0.05 = 0.06057					

Appendix Table 2.10. Two-way ANOVA for RGR.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	0.12589	0.04196	91.5**	2.72
temperature	2	0.00228	0.00114	2.48	3.11
Type	2	0.00989	0.00494	10.78**	3.11
Time	1	0.02153	0.02153	46.94**	3.96
Light dark cycle* temperature	6	0.01501	0.0025	5.45**	2.21
Light dark cycle* Type	6	0.00894	0.00149	3.25**	2.21
Temperature* type	4	0.0034	0.00085	1.85	2.49
Light dark cycle* Time	3	0.0252	0.0084	18.31**	2.72
Temperature* Time	2	0.01554	0.00777	16.94**	3.11
Type*time	2	0.00075	0.00038	0.82	3.11
Light-dark cycle* temperature* type	12	0.0051	0.00042	0.93	1.88
Light dark cycle* temperature* Time	6	0.0149	0.00248	5.41**	2.21
Light dark cycle* Type* Time	6	0.00148	0.00025	0.54	2.21
Temperature* type* time	4	0.00392	0.00098	2.14	2.94
Light dark cycle* temperature* type* time	12	0.01246	0.00104	2.26*	1.88
Block	1	0.0011	0.0011	2.41	3.96
Error	71	0.03256	0.00046		

Appendix Table 2.11. Leaf weight ratio (LWR) of three barley genotypes grown under different T cycles and temperatures.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	0.44994ABCD	0.40228ABCD	0.45796ABCD	0.53743A
	PDX	0.38533ABCD	0.37287BCD	0.41759ABCD	0.4702 ABCD
	GP	0.40444ABCD	0.41695ABCD	0.46024ABCD	0.50229ABCD
22°C	PDWT	0.44248ABCD	0.4509 ABCD	0.50404ABCD	0.4621 ABCD
	PDX	0.40185ABCD	0.47758ABCD	0.46058ABCD	0.42574ABCD
	GP	0.35132D	0.43123ABCD	0.42502ABCD	0.35922CD
27°C	PDWT	0.45068ABCD	0.46044ABCD	0.48222ABCD	0.49845ABCD
	PDX	0.41738ABCD	0.50107ABCD	0.44181ABCD	0.5234AB
	GP	0.3977 ABCD	0.48224ABCD	0.51683AB	0.51165ABC
LSD (interaction) α0.05 = 0.090749					

Appendix Table 2.12. Two-way ANOVA for LWR.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	0.00987	0.00329	3.85*	2.72
temperature	2	0.08151	0.04075	47.73 **	3.11
Type	2	0.00450	0.00225	2.64	3.11
Time	1	0.01166	0.01166	13.66**	3.96
Light dark cycle* temperature	6	0.04071	0.00678	7.95 **	2.21
Light dark cycle* Type	6	0.01065	0.00178	2.079	2.21
Temperature* type	4	0.01912	0.00478	5.598**	2.49
Light dark cycle* Time	3	0.03963	0.01321	15.47**	2.72
Temperature* Time	2	0.01067	0.00534	6.25**	3.11
Type*time	2	0.00331	0.00165	1.94	3.11
Light dark cycle* temperature* type	12	0.01049	0.00087	1.024	1.88
Light dark cycle* temperature* Time	6	0.02096	0.00349	4.093**	2.21
Light dark cycle* Type* Time	6	0.00513	0.00085	1.001	2.21
Temperature* type* time	4	0.00983	0.00246	2.879*	2.94
Light dark cycle*temperature* type*time	12	0.02373	0.00198	2.316	1.88
Block	1	0.00012	0.00012	0.144	3.96
Error	71	0.06061	0.00085		

Appendix Table 2.13. Unit leaf rates (ULR) of three barley genotypes grown under different T cycles and temperatures.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	0.001058ABCDEF	0.00124ABCDEF	0.000979BCDEF	0.001842ABCDE
	PDX	0.001048ABCDEF	0.001163ABCDEF	0.00084BCDEF	0.0019ABCD
	GP	0.000781BCDEF	0.001039ABCDEF	0.000975BCDEF	0.001886ABCDE
22°C	PDWT	0.000726CDEF	0.00066CDEF	0.00091BCDEF	0.002383A
	PDX	0.000643CDEF	0.00071CDEF	0.00054DEF	0.00213AB
	GP	0.000611CDEF	0.000509EF	0.000397F	0.00162ABCDEF
27°C	PDWT	0.001378ABCDEF	0.001207ABCDEF	0.001078ABCDEF	0.002141AB
	PDX	0.000837BCDEF	0.000999ABCDEF	0.000978BCDEF	0.001835ABCDE
	GP	0.000903BCDEF	0.00065CDEF	0.000719CDEF	0.001968ABC
LSD (interaction) α0.05 = 0.00093					

Appendix Table 2.14. Two-way ANOVA for ULR.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	4.462	1.4873	201.34**	2.72
temperature	2	0.033	0.0167	2.262	3.11
Type	2	0.176	0.0882	11.940*	3.11
Time	1	0.207	0.207	28.06**	3.96
Light dark cycle* temperature	6	0.292	0.0486	6.58**	2.21
Light dark cycle* Type	6	0.071	0.0118	1.596	2.21
Temperature* type	4	0.004	0.001	0.13	2.49
Light dark cycle* Time	3	0.354	0.1181	15.99**	2.72
Temperature* Time	2	0.334	0.167	22.61**	3.11
Type*time	2	0.033	0.0165	2.24	3.11
Light-dark cycle* temperature* type	12	0.048	0.004	0.545	1.88
Light dark cycle* temperature* Time	6	0.188	0.0313	4.24**	2.21
Light dark cycle* Type* Time	6	0.008	0.0013	0.171	2.21
Temperature* type* time	4	0.054	0.0136	1.84	2.94
Light dark cycle* temperature*type*time	12	0.205	0.0171	2.31*	1.88
Block	1	0.012	0.012	1.65	3.96
Error	71	0.524	0.0074		

Appendix Table 2.15. Leaf area ratio (LAR) of three barley genotypes grown under different T cycles and temperatures.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	138.61CDEFGHI	130.6DEFGHI	149.71BCDEFGHI	104.09FGHI
	PDX	132.00DEFGHI	148.33BCDEFGHI	129.79DEFGHI	100.1HI
	GP	158.96ABCDEFGHI	168.7ABCDEFGHI	128.9DEFGHI	108.9FGHI
22°C	PDWT	146.26BCDEFGHI	212.97ABC	183.33ABCDEF	127.55DEFGHI
	PDX	166.49ABCDEFGHI	220.99AB	179.98ABCDEFG	145.29BCDEFGHI
	GP	148.67BCDEFGHI	231.89A	166.14ABCDEFGHI	149.06BCDEFGHI
27°C	PDWT	129.02DEFGHI	175.27ABCDEFGH	182.23ABCDEF	90.25I
	PDX	150.74BCDEFGHI	189.86ABCDE	171.17ABCDEFGH	119.36EFGHI
	GP	180.6ABCDEFG	200.09ABCD	192.75ABCDE	100.94GHI
LSD (interaction) α0.05 = 53.03173					

Appendix Table 2.16. Two-way ANOVA for LAR.

Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	110903	36968	10.45**	2.72
temperature	2	15530	7765	22.75**	3.11
Type	2	1969	984	2.86	3.11
Time	1	124	124	0.362	3.96
Light dark cycle* temperature	6	10036	1673	4.86**	2.21
Light dark cycle* Type	6	3558	593	1.725	2.21
Temperature* type	4	3594	898	2.61*	2.49
Light dark cycle* Time	3	12858	4286	12.46	2.72
Temperature* Time	2	4392	2196	6.38**	3.11
Type*time	2	1351	676	1.96	3.11
Light dark cycle* temperature* type	12	3743	312	0.91	1.88
Light dark cycle* temperature*Time	6	3340	557	1.62	2.21
Light dark cycle* Type* Time	6	1774	296	0.86	2.21
Temperature* type* time	4	746	187	0.542	2.94
Light dark cycle* temperature* type* time	12	6806	567	1.65	1.88
Block	1	3	3	0.007	3.96
Error	71	24427	344		

Appendix Table 2.17. Specific leaf areas (SLA) of three barley genotypes grown under different T cycles and temperatures.

Temperature	Line	Photoperiod length			
		T20	T24	T30	Constant light
17°C	PDWT	316.88DEFGHIJKL	337.68DEFGHIJK	3.55DEFGHIJKL	197.26KL
	PDX	345.9DEFGHIJ	357.76CDEFGHI	307.1EFGHIJKL	225.28IJKL
	GP	349.26DEFGHIJ	409.83ABCDEF	338.65DEFGHIJK	222.16IJKL
22°C	PDWT	336.76DEFGHIJK	495.51ABC	378.19CDEFG	236.63GHIJKL
	PDX	417.67ABCDEF	533.94AB	431.73ABCDEF	232.09HIJKL
	GP	376.54CDEFGH	547.68A	462ABCD	286.77FGHIJKL
27°C	PDWT	306.19EFGHIJKL	416.71ABCDEF	383.75CDEF	190.64L
	PDX	331.04DEFGHIJKL	445.72ABCDE	398.64BCDEF	235.9GHIJKL
	GP	327.17DEFGHIJKL	459.52ABCD	386.73CDEF	207.38JKL
LSD (interaction) α0.05 = 96.98177					

Appendix Table 2.18. Two-way ANOVA for SLA.

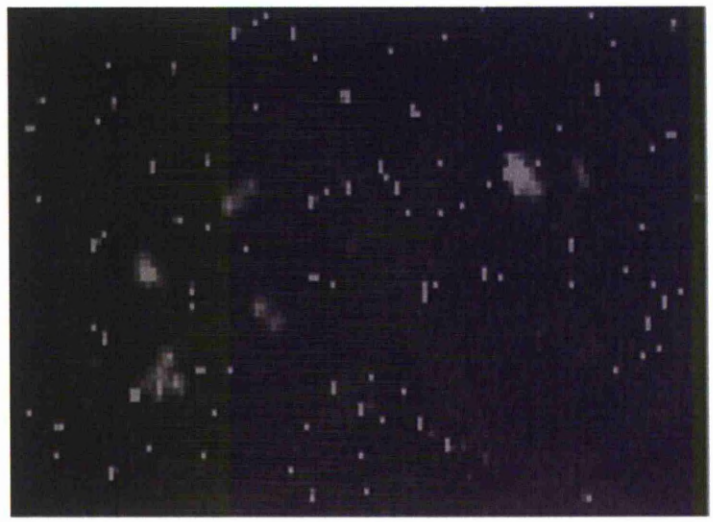
Source	DF	SS	MS	F	F≤0.05
Light dark cycle	3	709314	236438	186.27**	2.72
temperature	2	101734	50867	40.08**	3.11
Type	2	13045	6522	5.14**	3.11
time	1	11697	11697	9.215	3.96
Light dark cycle* temperature	6	37051	6175	4.87**	2.21
Light dark cycle* Type	6	9493	1582	1.25	2.21
Temperature* type	4	688	172	0.135	2.49
Light dark cycle* Time	3	23965	7988	6.29**	2.72
Temperature* Time	2	34913	17456	13.75**	3.11
Type*time	2	4423	2211	1.74	3.11
Light dark cycle* temperature* type*	12	19618	1635	1.29	1.88
Light dark cycle* temperature*Time	6	16689	2782	2.19	2.21
Light dark cycle* Type* Time	6	8018	1336	1.05	2.21
Temperature* type* time	4	3783	946	0.75	2.94
Light dark cycle* temperature* type* time	12	7751	646	0.51	1.88
Block	1	569	569	0.45	3.96
Error	71	90121	1269		

APPENDIX 3: Luciferase Gene

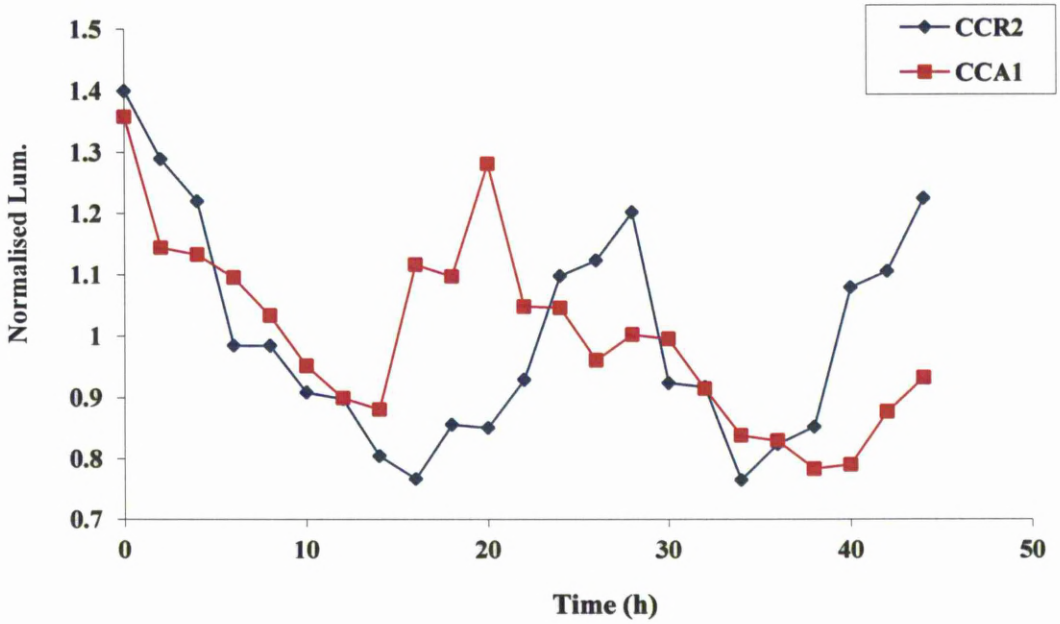
Using firefly Luciferase as reporter gene in barley

In recent years, the luciferase gene has been commonly used to display gene expression in the *Arabidopsis* circadian clock. For early detection of luciferase activity in barley plants, *Agrobacterium* strain GV3101 carrying pPCV812 binary vector, which has the *LUC+* marker gene driven by *Arabidopsis CIRCADIAN RHYTHM2* promoter (*CCR2::LUC+*) or *CIRCADIAN CLOCK ASSOCIATED1* promoter (*CCA1::LUC+*), was transferred into immature barley embryos. After the embryos were transferred to the transition medium, they were exposed for 20 mins to red/blue light at 2 h time intervals. The transformed embryos emitted a low level of light when supplied with luciferin (Figure 14). The results of this experiment indicated that rhythms of *Arabidopsis CCA1* fused to luciferase peaked at dawn while *CCR2* fused to luciferase peaked at dusk; and these findings are similar to the results obtained from *Arabidopsis* plants (Figure 15). The early observations showed that rhythmic expression of the *LUC+* marker in the barley embryos was slightly weak as a result of the *LUC+* gene being driven by the *Arabidopsis* promoter and without monocots' introns, and, as mentioned before, the *LUC+* gene was transferred by using *Agrobacterium* strain GV3101, which is not uncommonly used for barley transformation compared with *Agrobacterium* strain AGL1. Barley transformation protocol was original designed using Agrobacterium strain AGL1. Hence, after the embryos infected with Agrobacterium, they were transferred into induction medium contained Hygromycin as selective agents and Timentin to inhibit the growth of Agrobacterium cells during the embryogenic callus. On the other hand, using Agrobacterium strain GV3101 made it difficult to prevent the growth of the

Agrobacterium during the embryogenic callus thus most of the embryos died in early stage



Appendix Figure 3.1. Glow of transformed embryos with *Arabidopsis CCR2::LUC+* (on the left) and *Arabidopsis CCA1::LUC+* (on the right) constructs when supplied with luciferin.



Appendix Figure 3.2. Rhythms' expression of *Arabidopsis CCR2::LUC+* and *Arabidopsis CCA1::LUC* in barley embryos.